INSIGHTS INTO DISEASE RESISTANCE:
GENETIC ARCHITECTURE, GENES, AND PLEIOTROPY IN MAIZE

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INSIGHTS INTO DISEASE RESISTANCE: GENES, GENETIC ARCHITECTURE, AND PLEIOTROPY IN MAIZE

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The genes and mechanisms underlying quantitative disease resistance remain largely elusive. The objective of this dissertation was to resolve the structure of multiple disease resistance loci, explore the dynamics that shape the genome at those loci, and identify genes associated with plant defense. In order to do this, both locus-specific and genome-wide approaches were taken, as each resistance locus has a unique resistance profile and mechanism(s) of resistance. Bins 1.02 and 1.06 of the maize genome carry loci of interest conditioning multiple disease resistance. The two loci differ in allelic diversity, pathogen specificity, and mechanism of resistance. The locus in bin 1.06 is particularly interesting, as it has been characterized as yield-stabilizing and exhibits signs of genome plasticity. I have used fine-mapping, association mapping, expression evidence, and mutant analysis to dissect these loci, identify candidate genes, and demonstrate the role of candidate genes in plant defense. Each locus was unique, although common themes arose. Both loci may have multiple underlying genes, demonstrating that the genetic architecture of disease resistance is complex. Resistance to multiple diseases appears to be due to linkage, although there may be a role for pleiotropy at both loci. Fine-mapping narrowed the intervals, and was complemented by association mapping and expression analysis to evaluate candidate genes. A putative remorin was implicated by fine-mapping and expression analysis; *roughsheath2-interacting KH domain protein* (*rik*) and *pangloss1* (*pan1*)

were identified through fine-mapping and association mapping. *rik* was later eliminated as a candidate for the QTL of interest through fine-mapping and association mapping. Mutants were used to confirm the role of candidate genes in plant defense, including for *pan1* and the putative remorin. Based on these results, *pan1* was inferred to be a susceptibility gene for NLB and Stewart's wilt, and increased resistance was correlated with decreased expression. Susceptibility conditioned by wild-type *pan1* could be due to a passive mechanism, such as altered anatomical structures, or an active process, such as actin re-organization during pathogen attack. To test genome-wide association mapping candidate genes, mutants were identified and evaluated for NLB phenotype. Approximately 37% of the 123 families tested differed in disease phenotype from the background line. One of these was the putative remorin gene, which was inferred to contribute to resistance. Overall, I have examined candidate genes, explored genomic structure at these loci, and demonstrated a role for *pan1* in resistance to multiple diseases.

BIOGRAPHICAL SKETCH

Tiffany was born and raised in Upper Bucks County, Pennsylvania. She attended Moravian College in Bethlehem, PA. While doing an internship in a microbiology lab in Germany, her interests in host-microbial interactions and genetics were sparked. After graduating with a B.A. in German and a B.S. in Biology in 2008, she began her graduate studies at Cornell in 2008. While at Cornell her interests in plant breeding have developed and she has gained experience in international agriculture. She is fascinated by the intimate interaction between host and pathogen and the dynamics that shape the plant genome. During this time she met and married her husband, Santiago. Their son, Lucas, was born in 2011.

Dedicated to my husband, Santiago

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LIST OF ABBREVIATIONS

dQTL- disease quantitative trait locus

LRR- leucine rich repeat

GWAS- genome-wide association study

NIL- near-isogenic line

NLB- northern leaf blight

MDR- multiple disease resistance

QTL- quantitative trait locus

CHAPTER 1

THE GENETIC BASIS OF DISEASE RESISTANCE IN MAIZE[1]

Abstract

This chapter presents an overview of diseases important to global maize production, outlines the current understanding of the genetic underpinnings for resistance to these diseases, and explores how these findings can be used to improve maize. With a primary focus on fungal diseases, we review the current understanding of qualitative and quantitative resistance. In order to dissect the genetics of quantitative resistance to three important diseases, new datasets and resources have been utilized. A number of populations have been evaluated for various maize diseases, including biparental populations, association mapping panels, and the nested association mapping population. By generating lists of genes that are hypothesized to be involved in the interaction between plant and pathogen, both genome-wide association mapping and nested association mapping have provided hints about the biology of disease resistance. As part of the study of the architecture of disease resistance, both single disease resistance and multiple disease resistance have been explored. Multiple disease resistance is rare, but some genes apparently confer resistance to multiple pathogens. As high-resolution mapping becomes available, the challenge remains to translate this knowledge into breeding outcomes. Marker-assisted selection can be used to utilize these results, but there is a disconnect between the wealth of mapping information and the application of this data. Genomic selection is emerging as a powerful tool for maize improvement. The

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challenge, however, remains to apply mapping studies and basic biology to plant breeding to decrease the amount of maize lost to pathogens.

Introduction

Biotic stresses constrain maize production worldwide, affecting food security and prices. Demand is being spurred by population growth and the use of grain for biofuels, while supply is challenged by climate variability, as well as rising costs of fertilizer and water. Crop losses caused by maize diseases worldwide, excluding viruses, have been estimated at 4-14% (Oerke 2006). It is thus increasingly important to reduce losses due to diseases. While several pathogens cause grain yield losses, others contaminate maize seeds with mycotoxins, a widespread hazard to human and animal health (Wild and Gong 2010).

Because of maize's importance, its genetics and biology has been the focus of considerable research effort in the public and private sectors. A number of advanced breeding and genomic resources have been developed for understanding the genetics of resistance in maize, including populations derived from biparental crosses (e.g. Coe *et al.,* 2002; Szalma *et al.,* 2007; Belcher *et al.,* 2012), several association mapping panels (Flint-Garcia *et al.,* 2005; Yan *et al.,* 2011), a nested association mapping population (McMullen *et al.,* 2009; Yu *et al.,* 2008), and large genomic and sequence datasets (Ganal *et al.,* 2011; Gore *et al.,* 2009). These resources have been utilized, at least to some extent, to better understand the genetic architecture of resistance for multiple diseases (e.g. Kump *et al.,* 2011; Poland *et al.,* 2011; Wisser *et al.,* 2006; Wisser *et al.,* 2011).

With its advanced genetic and genomic resources, maize can be used both as a

model system for understanding plant-pathogen interactions, and as a practical system in which these basic biological findings can be applied in breeding programs to address farmers' production constraints. The challenge is to produce more resistant varieties in the context of various scientific and resource constraints and within the organization of the global maize breeding infrastructure. The purpose of this chapter is to summarize the current understanding of the genetic basis of disease resistance in maize and to note some of the challenges and frontiers in its application.

Understanding the intruders: diseases of maize

Historically, maize has suffered major losses due to disease, with perhaps the best-known epiphytotic being the southern leaf blight (SLB) epidemic caused by *Cochliobolus heterostrophus* in 1970-71 in the United States. At the time of the epidemic, the *T-urf13* gene conferring cytoplasmic male sterility was widely used in maize hybrid seed production. About 85% of the US maize crop carried this gene in 1970 (Ullstrup 1972). As it turned out, *T-urf13* also conferred specific hyper-susceptibility to a toxin produced by *C. heterostrophus* race T (Wise *et al.,* 1999). The ensuing SLB epidemic of 1970 was one of the most economically damaging plant disease epidemics of all time: yield loss over the USA for that season was estimated at 20-30%, with some areas suffering 50-100% losses (Ullstrup 1972). The amount of maize lost to the disease was much larger than, for instance, the amount of potato lost during the Irish late blight epidemic of the 1840s. Since susceptibility was under very simple genetic control, simply switching to germplasm lacking *T-urf13* was sufficient to rapidly control the disease in the following seasons. It should be noted that the cause of the epidemic was not an overall lack of

genetic diversity, but rather the ubiquity of a single disease susceptibility gene within elite germplasm.

Today, global maize diseases that pose threats to yield and human health include fungal diseases that attack the leaves, stem and ear (Balint-Kurti and Johal 2009). Globally important foliar diseases include southern leaf blight (SLB) caused by *C. heterostrophus*, southern rust caused by *Puccinia polysora*, common rust caused by *Puccinia sorghi*, northern leaf blight (NLB) caused by *Setosphaeria turcica*, and gray leaf spot (GLS) caused by *Cercospora zeae-maydis* and *Cercospora zeina.* Diplodia and Fusarium stalk and ear rots and Fusarium and Aspergillus kernel and ear rots are also important in many regions. Diseases of regional importance include tar spot complex (caused by *Phyllachora maydis* and *Monographella maydis*) in Latin America, and maize streak virus (MSV) in Africa (Shiferaw *et al.,* 2011). In addition to established diseases, emerging diseases, such as banded leaf and sheath blight (BLSB) in Asia (Pingali 2001) caused by *Rhizoctonia solani*, also pose potential future constraints to maize production. Maize diseases have been reviewed elsewhere more extensively (Balint-Kurti and Johal 2009; Pratt and Gordon 2006; White 1999).

The emergence of some diseases is related to changes in farming practices and production. For example, the increase in BLSB is correlated with an increase in maize production near rice paddies (Pingali 2001). *R. solani*, causal agent of BLSB, has a broad host range and isolates virulent on rice can also infect maize (Pascual *et al.,* 2000). Reduced tillage, which allows inoculum to overwinter in stalk debris on the soil surface and to re-infect maize the following season, has increased the distribution and severity of diseases such as GLS (Latterell and Rossi 1983; Ward *et al.,* 1999).

In some cases, agronomic techniques or biocides are used to manage diseases. Diversity at the population level can be used to suppress disease progress (e.g. Mundt 2002), so synthetic populations or other open-pollinated varieties may present opportunities for deployment of population-level genetic diversity for disease management. Varietal resistance is, however, the dominant and most convenient approach to disease management in crops in general, as well as in maize. Advances in precision agriculture are beginning to provide the opportunity to plant different varieties of maize in the same high-production fields. For purposes of this chapter, we consider genetic resistance in the context of analyzing or breeding individual genotypes. The overall genetic diversity available within maize is high compared to most crop species (Goodman 1983; Tenaillon *et al.,* 2001; Sachs *et al.,* 2009), and the primary gene pool is a rich source of disease resistance alleles for crop improvement. While the genetic diversity amongst elite hybrids is relatively low (Smith *et al.,* 1992; Romay *et al.*, 2013), diverse exotic germplasm can be utilized to identify and introduce novel disease resistance genes and alleles into maize varieties (Goodman 1999).

Understanding the system: Genetic architecture of disease resistance in maize and biological insights

Plant disease resistance is often categorized as qualitative (complete) or quantitative (partial) based on the extent of disease in a "resistant" interaction (Vanderplank 1968). While these categories are often presented as quite distinct, there is a gray area between these types of resistance in practice (Poland *et al.,* 2009; see below). A number of qualitative resistance genes (often referred to as major genes) have been cloned in a

number of different plant systems (e.g. Ellis *et al.,* 2000; Sanseverino *et al.,* 2010). Most major genes function by detecting the presence or the activity of pathogen-derived proteins and then inducing a rapid, localized defense response (called a hypersensitive response) at the point of infection, which limits pathogen growth (Bent and Mackey 2007). This type of defense is often referred to as effector-triggered immunity or ETI (Jones and Dangl 2006), and major genes of this type have been referred to as R-genes. R-genes generally provide high levels of resistance and are easy to manipulate in breeding programs. However, they generally provide only race-specific resistance and are often easily overcome by the pathogen, such that they are generally not durable in an agricultural context (McDonald and Linde 2002).

At least 17 qualitative resistance genes have been identified and mapped for several diverse maize diseases, including maize streak virus, NLB, and southern and common rust (Wisser *et al.,* 2006). Four of these genes (*Rp1*, *Rp3*, *Rxo1*, and *Hm1*) have been cloned. *Rp1*, *Rp3*, and *Rxo1* all carry the domain typical of R-genes, the nucleotide binding site-leucine rich repeat (NBS-LRR) (Collins *et al.,* 1999; Webb *et al.,* 2002; Zhao *et al.,* 2005). *Rp1* and *Rp3* confer resistance to specific races of common rust while *Rxo1* confers resistance to bacterial stripe of maize. *Hm1*, which confers resistance to *Cochliobolus carbonum* race 1, encodes an NADPH-dependent HC-toxin reductase that detoxifies HC-toxin produced by the fungus (Johal and Briggs 1992). Interestingly, the *Hm1* gene is extremely widespread in maize and consequently only a few lines that lack the *Hm1* gene are susceptible to *C. carbonum* race 1. Furthermore, genes with high homology to *Hm1* are present in all grass genomes tested. Specific silencing of the *Hm1* homolog in barley rendered the plant susceptible to *C. carbonum* race 1. *Hm1* appears to

have evolved early in the grass lineage, possibly under selection for resistance to HC-toxin (Sindhu *et al.,* 2008).

Quantitative, or partial, disease resistance is generally controlled by multiple loci, each with relatively small effects. In general, this form of resistance is more durable in the field than qualitative resistance and is therefore agronomically important (McDonald and Linde 2002). The underlying mechanisms associated with quantitative disease resistance in plants are not well understood. To date, the identity of five quantitative genes or gene clusters associated with disease resistance in plants have been determined (Broglie *et al.,* 2006; Fu *et al.,* 2009; Fukuoka *et al.,* 2009; Krattinger *et al.,* 2009; Manosalva *et al.,* 2009). These genes appear to be unrelated and confer resistance by a variety of mechanisms, although these mechanisms are not entirely clear at this point. They include an NBS-LRR gene (Broglie *et al.,* 2006), a START kinase (Fu *et al.,* 2009), an ABC transporter (Krattinger *et al.,* 2009), a proline-rich protein of unknown function (Fukuoka *et al.,* 2009) and a family of germin-like proteins (Manosalva *et al.,* 2009). This diversity of gene classes is consistent with the emerging consensus that variation in quantitative disease resistance in plants is likely based on variation in genes involved in a number of different mechanisms and pathways (Kliebenstein and Rowe 2009; Poland *et al.,* 2009).

Generally, disease resistance quantitative trait loci (dQTL) are thought to be race non-specific (Vanderplank 1968), but there are multiple examples of race-specific QTL (e.g. Kolmer and Leonard 1986; Leonards-Schippers *et al.,* 1994; Marcel *et al.,* 2008; Qi *et al.,* 1999; Talukder *et al.,* 2004). Therefore, to ensure the effective deployment of dQTL it is important to assess the effectiveness of the resistance with respect to the

pathogen populations against which the resistance is intended to perform. Preliminary assessments can be made by testing source germplasm and/or derived lines with pathogen isolates considered to represent the target population. Candidate germplasm should be tested over a number of different environments to ensure as much as possible that the resistance is broadly effective.

Numerous dQTL studies in maize have been carried out. Genotypic variation has been associated with variation in resistance to all classes of disease, including viral, bacterial, and fungal leaf blights, ear rots and stalk rots (e.g. Ali *et al.,* 2005; Brown *et al.,* 2001; McMullen *et al.,* 1994; Ming *et al.,* 1997; Paul *et al.,* 2003; Pernet *et al.,* 1999; Robertson-Hoyt *et al.,* 2006; Xia *et al.,* 1999). A synthesis of 50 studies reporting the locations of 437 QTL associated with resistance to 19 maize diseases identified QTL on both arms of all 10 maize chromosomes (Wisser *et al.,* 2006). The composite QTL map showed 89% of the maize genome to be associated with disease resistance, reflecting the low resolution of the mapping procedures employed, as well as indicating that there are large numbers of dQTL in the maize genome.

In recent years, a number of resources and datasets have been generated to gain a more precise idea of the genetic architecture underlying quantitative disease resistance in maize, as shown in Table 1. Maize is generally well-suited for association mapping (Yan *et al.,* 2011) due to the high genetic diversity among lines (Liu *et al.,* 2003). The generally low levels of linkage disequilibrium found in maize (Remington *et al.,* 2001; Chia *et al.*, 2012) mean that, given an appropriate population and accurate genotypic and phenotypic data, association mapping has the potential to resolve QTL to their causal genes and potentially nucleotides. A number of maize association mapping populations

have been developed in the public sector, including the 300-line Goodman panel (Flint-Garcia *et al.,* 2005) mentioned below that has been evaluated for NLB, SLB, and GLS (Wisser *et al.,* 2011). Association mapping in maize was formerly limited to the analysis of candidate genes (i.e. genes already suspected of being important in controlling variation for the trait of interest) (Harjes *et al.,* 2008; Krill *et al.,* 2010; Wilson *et al.,* 2004). The increasing quantity of genotypic information now permits genome-wide association studies (GWAS), in which the entire genome is scanned for marker-trait associations in a relatively unbiased way (Belo *et al.,* 2008; Cook *et al.,* 2012).

There are however issues with association mapping in maize, including fast LD decay, a large number of alleles, and many rare alleles, as shown in Table 1. Another difficulty with GWAS is that the multiple test corrections associated with the very large number of tests conducted lead to very high significance thresholds, such that even for traits with high heritabilities, few significant associations may be identified. In a GWAS of kernel starch, protein, and oil traits, with broad-sense heritabilities ranging from 83% to 91%, no significant associations were identified after multiple test corrections (Cook *et al.,* 2012). Significant or otherwise, intriguing GWAS associations need to be validated independently, for instance using mutants, transgenics and/or fine-mapping studies.

Another breeding tool utilized to understand the genetic architecture of disease resistance in maize is the nested association mapping (NAM) population (McMullen *et al.,* 2009; Yu *et al.,* 2008). The NAM population consists of 25 linked recombinant inbred populations of 200 lines each. Each of these populations is derived from a cross between B73 and one of a set of 25 diverse lines. Analyzed as a single population, the NAM population has unprecedented mapping power due to its large size (5,000 lines) and

the effective combination of linkage and linkage-disequilibrium approaches (Yu *et al.,* 2008). This, in theory, allows resolution to the single gene level (Cook *et al.,* 2012; Poland *et al.,* 2011; Tian *et al.,* 2011). The NAM population has been evaluated for SLB, NLB and GLS (Benson *et al.,* 2011; Kump *et al.,* 2011; Poland *et al.,* 2011). The genetic architectures controlling variation in resistance to SLB and NLB were found to be broadly similar: 32 and 29 dQTL were identified for the two diseases respectively. These dQTL were of relatively small effect and no epistatic interactions were identified. In these respects, the genetic architectures controlling variation in SLB and NLB resistance were similar to those controlling other quantitative traits, including flowering time and various leaf and kernel composition traits, that have been analyzed in this population (Buckler *et al.,* 2009; Cook *et al.,* 2012; Tian *et al.,* 2011). The genetic architecture of gray leaf spot differed, with fewer (16) QTL identified and epistasis was detected (Benson 2013).

The results of NAM GWAS provide a preliminary look at the genes that may underlie the trait of quantitative disease resistance. GWAS revealed more than 1,000 associations with specific SNPs for SLB and NLB resistance traits in the NAM population (Chia *et al.*, 2012, Kump *et al.*, 2011, Poland *et al.*, 2011). It is likely that in many cases the SNPs identified are at or very near to the actual causal genes (Cook *et al.,* 2012; Tian *et al.,* 2011). For both NLB and SLB, many of the associated SNPs were within or adjacent to genes which have been previously implicated in disease resistance or the defense response. For NLB, genes implicated by GWAS included many defense candidates including those encoding serine-threonine protein kinases, receptor-like kinases, antifreeze proteins, a germin protein, and an ABC transporter, among others.

Results were similar for SLB, with candidate genes including those encoding serine-threonine kinases, an ABC transporter, a GST, and an LRR receptor kinase, among others. The identification of receptor-like kinases as candidate genes for quantitative resistance loci for both diseases is consistent with the hypothesis that modest levels of resistance are associated with host recognition of conserved pathogen features. Recognition of "pathogen-associated molecular patterns" (PAMPs) has been linked to disease resistance in several cases and is associated with partial restriction of pathogen infection (Bent and Mackey 2007). As noted above, genes implicated by GWAS need to be confirmed with complementary evidence.

There are several lines of evidence suggesting that some loci may condition resistance to more than one disease (reviewed by Poland *et al.,* 2009; Kou and Wang 2010; Krattinger *et al.,* 2009). Loci conditioning multiple disease resistance (MDR) would make breeding for disease resistance more efficient. In synthesizing the results of 50 mapping studies, Wisser *et al.,* (2006) found that dQTL were non-randomly distributed in the maize genome. At several loci, dQTL for different diseases were clustered, suggesting the presence of genes conferring multiple disease resistance. Other QTL mapping studies and analyses of introgression lines have provided additional evidence for the existence of MDR genes and loci in maize with respect to a variety of disease combinations (Balint-Kurti *et al.,* 2010; Belcher *et al.,* 2012; Chung *et al.,* 2011; Kerns *et al.,* 1999; Welz *et al.,* 1999; Zwonitzer *et al.,* 2010).

Questions remain as to whether the observed MDR is due to linkage or pleiotropy and whether MDR is the rule or the exception. To address these questions, we examined MDR to three foliar diseases of maize: GLS, SLB and NLB. These three diseases are

caused by fungi in the class Dothideomycetes and share somewhat similar modes of pathogenesis (Beckman and Payne 1982; Jennings 1957). It may be that genes conferring MDR target aspects of the pathogenesis process that are shared amongst these pathogens or pathogens are targeting 'hub' plant proteins (Mukhtar *et al.* 2011). Analyzing the disease ratings for an association panel of 300 diverse lines, Wisser *et al.* (2011) observed significant genotypic correlations between resistances to the three foliar diseases of maize, supporting the MDR hypothesis. Using an initial dataset of 858 SNPs, these authors reported the association of a glutathione-S-transferase gene with resistance to the three diseases.

Poland (2010) analyzed the correlations of disease ratings for these same diseases among the NAM founders (the 26 parents of the population), across the 5,000 lines of the population, and within the 25 individual families comprising the population. Correlations between the diseases were the highest within the diverse inbred founders, followed by the correlations among the 5,000 recombinant inbred lines (RILs), and correlations within RIL populations were the weakest. The modest disease correlations within RIL populations were not strongly supportive of the MDR hypothesis, suggesting instead that a large proportion of the strong correlations among parental lines could be due to the fact that some of the parental lines (those bred for disease-conducive environments) carry sets of resistance loci for multiple diseases.

A comparison of the QTL identified for SLB, NLB and GLS resistance in the NAM population allows a fairly explicit examination of the MDR hypothesis. A comparison of QTL and SNPs provides evidence for some loci with pleiotropic effects. In the NAM population, 23 genetic positions were identified for which quantitative

resistance loci for two or more diseases co-localized. At these loci, the estimated allele effects from each founder inbred were compared. At seven of these loci, allele effects were positively correlated, as would be expected for MDR genes or loci (Poland 2010). When GWAS results for NLB and SLB were compared, three genes (a predicted leucine zipper transcription factor and two unknown proteins) were identified as carrying SNP loci with significant associations with resistance to both diseases (Kump *et al.,* 2011; Poland *et al.,* 2011).

While there is evidence that some individual loci confer resistance to more than one disease, this phenomenon does not apparently explain the wider trends of pleiotropic QTL and correlated resistances. From our work to date, it seems clear that, at least with respect to SLB, GLS and NLB, most of the genetic disease resistance and in particular most of the dQTL of larger effect that we observe are disease-specific (Zwonitzer *et al.,* 2010). While a number of lines of evidence suggest the presence of MDR genes conferring resistance to SLB, GLS and NLB, it seems likely that many of these MDR loci individually have relatively small effects and may be below the detection threshold as individual loci (Balint-Kurti *et al.,* 2010).

Both in terms of understanding the nature of resistance and in choosing loci with complementary functions, it would be desirable to know the ways in which different QTL influence the process of pathogenesis. Analysis of specific dQTL using near-isogenic lines (NILs) differing only for a specific locus permits a better understanding of their quantitative and qualitative phenotypes than can be achieved in segregating families. NILs can be used to validate QTL, test association mapping hits, and characterize QTL. Characterization of two NLB QTL on chromosome 1 revealed that they influenced the

pathogenesis process in distinct ways. The QTL in bin 6 reduced the pathogen's success during the initial stages of infection, while the QTL in bin 2 reduced the extent of the pathogen's invasion of the leaf vasculature (Chung *et al.,* 2010).

Another important question about the agronomic use of disease resistance concerns the trade-offs that may exist with other traits. There are several examples from other plant systems in which the presence of genes associated with both qualitative and quantitative disease resistance incur a yield cost (Heidel *et al.,* 2004; Orgil *et al.,* 2007; Tian *et al.,* 2003; Todesco *et al.,* 2010). The only study addressing this question in maize thus far has been on the yield costs associated with the large-effect dQTL *Rcg1* for anthracnose stalk rot resistance (Frey *et al.,* 2011). In this case NILs were used to show that there are no fitness costs associated with *Rcg1* in non-diseased conditions and there is a yield benefit associated with *Rcg1* under inoculated conditions (Frey *et al.,* 2011).

Translating knowledge to action: breeding for disease resistance

As new resources and technologies permit the identification of dQTL with unprecedented precision, a key challenge will be translating the knowledge gained from mapping studies into breeding outcomes. This can be achieved through marker-assisted selection (MAS), including genomic selection (GS), and/or cisgenesis (direct transfer within species). Marker-assisted backcrossing and "forward crossing" are well-suited for the manipulation of genes with large effects (Holland 2004), while genomic selection is proving particularly useful for improving on traits with low heritability and/or under polygenic control (Heffner *et al.,* 2009).

In the past, the low resolution of mapping results meant that recombination could

readily separate the marker being used for selection from the desired allele. The use of "perfect" markers (those targeting the polymorphism that causes the phenotype of interest) obviously avoids this problem (Lande and Thompson 1990). As we know more about the genetic architecture of disease resistance in maize, we come closer to having "perfect" markers. Historically, there has been a trade-off between conventional breeding approaches and MAS in terms of cost and time, with MAS being faster but more costly, and conventional breeding schemes being slower but cheaper (Morris *et al.,* 2003). This led breeding efforts with constrained budgets, including many public breeding programs, to focus on conventional breeding schemes (Morris *et al.,* 2003). However, this is changing as marker technologies improve and genotyping costs decline. In one recent study, MAS in maize was shown to be more cost-effective than phenotypic selection when selecting for resistance to multiple foliar pathogens (Asea *et al.,* 2011) and for maize streak virus (Abalo *et al.,* 2009). The advantages of MAS will become more compelling as genotyping costs continue to decline and as more useful trait-marker associations become available for selection. Challenges associated with the effective deployment of MAS have been discussed in several recent reviews (Holland 2004; Hospital 2009; Johnson 2004).

When a trait is controlled by multiple QTL, or when multiple traits are being considered in a breeding program, a form of MAS known as genomic selection (GS) can be employed (Goddard and Hayes 2007; Meuwissen *et al.,* 2001). In GS, specific loci associated with a trait are not identified and selected for, but instead the effect of every marker is fitted as an effect in a model and used to make selections. The availability of low-cost, high-throughput genotyping methods has made GS a feasible and attractive

form of MAS. Using simulations, GS was predicted to result in up to a 43% greater genetic gain over marker-assisted recurrent selection, depending on levels of heritability and number of QTL (Bernando and Yu 2007). For polygenic traits with low heritability in maize, both GS and MARS outperformed phenotypic selection in terms of genetic gains (Bernardo and Yu 2007). GS has the potential to improve disease resistance traits in plants. Its use has been proposed for achieving durable stem rust in wheat (Rutkoski *et al.,* 2011), and it has the potential to increase genetic gain for traits with low heritability (Heffner *et al.,* 2009). Thus, GS could improve gains for resistance to maize diseases, including those such as *Aspergillus* ear rot and aflatoxin accumulation, for which it is notoriously difficult to achieve genetic gains and for which dQTL have small effects and are highly influenced by the environment (Brooks *et al.,* 2005; Paul *et al.,* 2003; Warburton *et al.,* 2009).

GS is gaining favor in maize breeding as high-throughput genotyping and extensive phenotypic datasets are generated. Since GS does not require the identification and careful characterization of loci and genes associated with variation in traits of interests, it may seem that QTL identification and characterization (previously seen as the basis of MAS) is now unnecessary. However, GS is only beginning to be proven, and basic science, plant pathology, and QTL mapping inform breeding programs (including GS programs) in several ways. These include understanding the mechanisms of resistance, improving phenotyping methods, and identifying sources of diverse alleles. An understanding of the mechanisms of resistance associated with specific dQTL can be used to predict complementary combinations of loci and alleles. For instance, knowing that one dQTL is associated with variation in susceptibility to penetration, while another

is associated with resistance to vascular invasion (Chung *et al.,* 2010), can allow a breeder to target both stages of pathogenesis by selecting for both QTL. Analysis of phenotyping methods, and development of new phenotypic assays, can also enhance breeding efficiency. For example, a method developed by Mideros *et al.,* (2009) to estimate *A. flavus* biomass by qPCR enables the separation of components of resistance such as fungal infection in multiple tissues and aflatoxin accumulation (Mideros 2012). By breeding for components of a trait, gains can be made.

By identifying and characterizing new, diverse alleles at mapped loci and characterizing the effects they can have on disease, public sector research into the genetic architecture of disease resistance can prove useful for breeders. The NAM analysis allowed outstanding alleles to be identified across a relatively broad set of maize germplasm that may not be present in private breeding programs. These insights might contribute to the strategic selection of parents in a breeding program, as the incorporation of known sources of resistance is essential to the success of GS for disease resistance (Rutkoski *et al.,* 2011). It is important to keep in mind that major genes can mask the effects of minor genes in GS scenarios, such that quantitative resistance is not selected upon (Rutkoski *et al.,* 2011). When this occurs, breeders could select for resistance-associated loci based on prior knowledge. Basic research has and will continue to define major and minor gene loci and these data can be incorporated into GS algorithms. Public mapping efforts to identify causative genes and polymorphisms can provide a basis for markers to include in genomic selection models. In addition, while GS populations may not be evaluated specifically for some diseases and traits, it is desirable to include resistance for these diseases.

Disease resistance has long been and remains an attractive target trait for genetically modified crops (Godfray *et al.,* 2010). Despite significant resources devoted to this area, few commercially viable plants with transgenically-conferred disease resistance traits are available; the exception are a few virus resistance traits (e.g. Gonsalves 1998). This is due to a combination of factors. Biological considerations include small allele effects, narrow spectra and potentially short durability of certain transgenically-conferred disease resistance traits as well as their yield costs (see above; Hammond-Kosack and Parker 2003). Non-biological considerations include the cost of developing transgenic lines, the availability of specific intellectual property, and public opposition to the deployment of the technology. New insights concerning plant quantitative resistance, effectoromics, plant basal defense mechanisms (Lacombe *et al.,* 2010) and mechanisms and applications of RNA interference (Nowara *et al.,* 2010; Wulff *et al.,* 2011; Yin *et al.,* 2011), however, are likely to lead to some practical applications in the foreseeable future. As more disease resistance alleles are cloned, it may be increasingly feasible to use these alleles via direct gene transfer among maize lines. Transfer of genes within different members of the Poaceae can also be effective: the maize NBS-LRR gene *Rxo1* protects rice against *Xanthomonas oryzae* pv. *oryzae* (Zhao *et al.,* 2005).

Another potential impact of transgenics in maize is the reduction of mycotoxins due to pest resistance provided by transgenic expression of toxins from *Bacillus thuringiensis* (Bt). Bt maize is thought to decrease mycotoxin accumulation because insect damage provides an entry point for the fungus and limiting insect damage in turn decreases mycotoxin accumulation (Dowd 2001). Lower levels of fumonisin have been

associated with the use of Bt maize (Hammond *et al.,* 2004). In 2004, the reduction of fumonisin and deoxynivalenol damage through the use of Bt maize was estimated to have an annual economic impact of $17 million dollars (Wu *et al.,* 2004). Bt maize has been shown to reduce aflatoxin accumulation when insect pressure is high (Williams *et al.,* 2002; Williams *et al.,* 2005; Windham *et al.,* 1999; Wu 2006).

Conclusions

To meet increasing demand for maize, yield constraints must be overcome. Biotic stresses pose an important constraint in many parts of the world, including Asia, Sub-Saharan Africa and Latin America (Pingali 2001). A number of new genomics-based resources have been developed for public maize research in the past few years, and have been employed to better elucidate disease resistance in maize. As a result, the genetic architecture and biology of resistance is better understood, but the challenge remains to translate this knowledge into improved disease resistance in maize varieties.

Understanding the biology of disease resistance can inform the search for genes effective in conditioning resistance, and thus contribute to harnessing genetic diversity for crop improvement. Loci conferring resistance to multiple pathogens are of particular interest and have been identified, but are relatively rare in maize. It will be of fundamental and practical interest to understand the mechanisms underlying such resistance and whether there are associated pleiotropic effects affecting other important agronomic traits, such as yield. An understanding of the multiple functions of defense-related genes can thus inform breeding decisions. Understanding the genetic architecture and biochemical pathways that underlie disease resistance will provide a route by which

to do so.

Table 1. Resources. Resources and tools used to identify QTL and genes, including some of the advantages and disadvantages associated with different methods.

Resource	Advantage	Disadvantage
Bi-parental linkage mapping	-Map QTL with diverse lines -Detect rare population-level alleles/QTL	-Poor mapping resolution
Nested association mapping	-Greater statistical power -Ability to detect small-effect QTL -Narrow QTL intervals possible	-All relative to a reference line -Large investment to make population -May not detect rare alleles -Genomic differences in reference line may limit recombination
Association mapping	-Many alleles examined -Smaller investment to make population -Nucleotide level resolution possible	-Many alleles in maize, including a large number of rare alleles -Population structure -False positives
Fine-mapping	-Higher resolution -Map QTL with diverse lines, and rare population-level alleles/QTL	-Low recombination or small allele effects can limit the resolution
Near-isogenic lines	-Background effects are controlled -Allele effects can be examined in different backgrounds	-Limited power to detect QTL with small effects -Many genes within QTL intervals
Mutant analysis	-Can examine effect of single genes on phenotype	-Natural allelic variation may not extend to null alleles
Expression analysis	-Individual genes involved in the response to a stimulus are discovered -Can detect novel genes/alleles in populations of interest	-Large amounts of data/candidates -Trait-controlling genes with structural differences are not reflected and may be passed over in expression data
Meta-analysis	-Increased resolution over individual bi-parental populations	-Curating data may be difficult and time-consuming

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CHAPTER 2

UNRAVELING GENOMIC COMPLEXITY AT A QUANTITATIVE DISEASE RESISTANCE LOCUS IN MAIZE IMPLICATES STRUCTURAL VARIATION AND THE RECEPTOR-LIKE KINASE *PAN1*[2]

Abstract

Multiple disease resistance (MDR) has important implications for plant fitness, given the selection pressure that many pathogens exert directly on natural plant populations and indirectly via variety improvement programs on crop plants. Evidence of a locus conditioning resistance to multiple pathogens was found in bin 1.06 of the maize genome with the allele from inbred line 'Tx303' conditioning quantitative resistance to northern leaf blight (NLB) and qualitative resistance to Stewart's wilt. To dissect the genetic basis of MDR in this region and to refine candidate gene hypotheses, we mapped resistance to the two diseases. Both resistance phenotypes were localized to overlapping regions, with the Stewart's wilt interval refined to a 95.9-kb segment containing three genes, and the NLB interval to a 3.60-Mb segment containing 117 genes. Regions of the introgression showed little to no recombination, suggesting structural differences between the inbred lines 'Tx303' and 'B73', the parents of the fine-mapping population. We examined copy number variation across the region using next-generation sequencing data and found large variation in read depth in 'Tx303' across the region relative to the reference genome of inbred line 'B73'. In the

[2] Jamann T, Poland J, Kolkman J, Smith L, and Nelson R (2014) Unraveling genomic complexity at a quantitative resistance locus implicates structural variation and *pan1* receptor-like kinase in resistance to multiple diseases in maize. GENETICS. Submitted.

fine-mapping region, association mapping for NLB implicated a few candidate genes, including a putative zinc finger and *pan1*. We tested mutant alleles and found that *pan1* is a susceptibility gene for NLB and Stewart's wilt. Our data strongly suggest that structural variation plays an important role in resistance conditioned by this region, and *pan1*, a gene conditioning susceptibility for NLB, may underlie the QTL.

Introduction

The genes and loci that influence host-pathogen interactions vary in allele effects, specificities, and linkage relationships. While disease resistance can be conditioned by single genes with large effect (Bent 1996, Jones and Dangl 2006), the emerging model of resistance for many plant diseases is complex in nature, with many genes and loci functioning in concert and each contributing a small proportion of the total phenotypic variation (Kump *et al.,* 2011, Poland *et al.,* 2011, Cook *et al.,* 2012). Each locus has a unique profile, with some loci contributing broad-spectrum protection against diverse pathogen species and strains. Investigating these intricacies offers the opportunity to understand the diverse ways in which plants defend themselves against microbial assault.

Correlated responses to multiple diseases have been observed in various germplasm panels, implying that there are loci and genes that condition broad-spectrum resistance (Rossi *et al.,* 2006, Gurung *et al.,* 2009, Wisser *et al.,* 2011). At the chromosomal segment level, disease and insect resistance loci co-localize in a non-random fashion (McMullen and Simcox 1995, Williams 2003, Wisser *et al.,* 2005) and loci have been identified that confer resistance to diverse pathogen isolates and taxa

(Zwonitzer *et al.,* 2010, Chung *et al.,* 2011, Belcher *et al.,* 2012). There is evidence to suggest that gene clusters can confer resistance to more than one disease. A cluster of germin-like proteins confers resistance to rice blast and sheath blight of rice (Manosalva *et al.,* 2009). Similarly, resistance gene homologs, which are known to co-localize with broad-spectrum disease resistance loci, can cluster in the genome and contribute a diversity of specificities (Lopez *et al.,* 2003, Ramalingam *et al.,* 2003). Pleiotropy remains uncommon in maize, and correlated responses may be due to linkage or population structure (Wallace *et al.,* 2014), although in some cases, individual genes have been shown to condition MDR. For example, the putative ABC transporter *Lr34* of wheat provides protection against leaf rust, stripe rust, and powdery mildew (Krattinger *et al.,* 2009). Pattern recognition receptors are able to detect molecular patterns from diverse organisms to confer disease resistance (Zipfel and Rathjen 2008).

While in some cases single genes or alleles common across diverse germplasm confer disease resistance, increasingly, the role of structural variation in plants is being explored and its effects on phenotypic variation recognized (Springer *et al.,* 2009, Chia *et al.,* 2012, McHale *et al.,* 2012). As quantitative trait loci (QTL) are subjected to fine-mapping, some loci fractionate into many QTL, each conditioned by one or more genes (Steinmetz *et al.,* 2002, Studer and Doebley 2011, Johnson, Haggard *et al.,* 2012). In some cases, the allele effect conditioned by each QTL is small enough that the individual locus cannot be identified in isolation (Buckler *et al.,* 2009, Poland *et al.,* 2011). In other cases, single resistance loci, such as *Rhg1*, are conditioned by multiple genes present in varying copy numbers in different lines (Cook *et al.,* 2012,

Maron *et al.,* 2013). Whole-genome studies have in fact suggested that structural variation is generally associated with disease resistance: structural variation in plants co-localizes with resistance nucleotide-binding proteins, receptor-like proteins, and disease resistance QTL (Lai *et al.,* 2010, McHale *et al.,* 2012, Xu *et al.,* 2012).

The conventional approach of genetic isolation and transgenic complementation remains the gold standard for demonstrating the function of a gene. This approach, however, is proving inadequate for dealing with the complexity underlying some loci, particularly for structural variation. Strong evidence for the importance of copy number variation in explaining trait variation (Cook *et al.,* 2012, Maron *et al.,* 2013) and the emerging model of plant defense with many loci each contributing a small effect combine to challenge this paradigm (Kump *et al.,* 2011, Poland *et al.,* 2011, Cook *et al.,* 2012). There is a need for a new approach that can take advantage of whole genome analyses, address presence/absence variation, and examine loci with small effects. This study represents such an approach and provides insights into a genetically complex locus affecting diverse traits.

In maize, chromosomal bin 1.06 has been identified as a key locus for stabilizing yield under adverse conditions, including both biotic and abiotic stress (Landi *et al.,* 2002, Tuberosa *et al.,* 2002, Landi *et al.,* 2010). In addition to plant architectural traits and yield under abiotic stress, resistance to many diseases has been localized to bin 1.06, including northern leaf blight (NLB), Stewart's wilt, southern leaf blight (SLB), common rust, grey leaf spot (GLS), and ear and stalk rot caused by multiple fungi (Wisser *et al.,* 2006, Chung *et al.,* 2010, Zwonitzer *et al.,* 2010). In a QTL study of the recombinant inbred line (RIL) population Ki14 x B73 evaluated for

three foliar fungal diseases, NLB, GLS, and SLB, a 33 Mb region spanning bins 1.05 and 1.06 was the only locus identified that conferred resistance to all three diseases (Zwonitzer *et al.,* 2010). A number of QTL studies for NLB resistance in maize have identified QTL at bin 1.06, ranging in physical size from 3 to 30 Mb (Freymark *et al.,* 1993, Welz *et al.,* 1999, Wisser *et al.,* 2006, Chung *et al.,* 2010, Van Esbroeck *et al.,* 2010, Chung *et al.,* 2011, Poland *et al.,* 2011). Additionally, bin 1.06 harbors the dominant Stewart's wilt resistance gene *Sw1* (Ming *et al.,* 1999).

Both NLB, caused by the fungus *Setosphaeria turcica*, and Stewart's wilt, caused by the bacterium *Pantoea stewartii*, are foliar, hemibiotrophic diseases important to maize production. Both pathogens spread through the vascular tissue, causing wilted lesions by plugging xylem vessels (Jennings and Ullstrup 1957, Roper 2011). The importance of genes localized to maize bin 1.06 in resistance to both NLB and Stewart's wilt has been described in multiple mapping populations. Using a population of Tx303 x B73 introgression lines (Szalma *et al.,* 2007), Chung *et al.,* (2010) showed that the NLB resistance QTL at 1.06 protects against fungal penetration.

To explore the genomic complexity of this important region, we constructed high resolution mapping populations at this locus and evaluated NLB and Stewart's wilt resistance using a set of Tx303 x B73 near-isogenic lines (Szalma *et al.,* 2007, Chung *et al.,* 2010). Fine-mapping allowed us to dissect the linkage relationship between the major-effect Stewart's wilt QTL and the minor-effect NLB QTL and to identify candidate genes. Using association mapping, we further refined the list of candidate genes for NLB resistance and using mutants confirmed a role for the

receptor-like kinase, *pan1*, in plant defense. Furthermore, multiple lines of evidence indicated a lack of genomic stability at the region, including reduced recombination across portions of the fine-mapping region in the NIL population and indicators of copy number variation.

Materials and methods

Plant materials

Fine-mapping

NILs used for fine-mapping were derived from the TBBC3 (**T**x303 x **B**73 **B**ack**c**ross **3**) population, a set of chromosomal segment substitution lines with Tx303 introgressions in a B73 background (Szalma *et al.,* 2007, Chung *et al.,* 2010). Chung *et al.* (2010) identified families TBBC3-38 and TBBC3-39, both with introgressions in 1.06, as significantly more resistant than B73. Selected families developed from these lines were chosen for fine- mapping: TBBC3-38_19E, TBBC3-38_15G, and TBBC3-38_17A (Chung *et al.,* 2010). The details of population development and evaluation are shown in Fig. 1. Briefly, a population was developed by crossing TBBC3-38_19E to B73. In the F_2 generation, 435 individuals were screened for recombinants. A total of 113 recombinant plants were identified, but seed was available from only 100 plants. Seed from 15 heterozygous F_2 individuals was advanced to the F_3 generation to screen for additional recombinants. A population of 4,080 F_3 seeds was planted and 2,929 plants were screened with flanking markers snp_01_0042 (180,394,924 AGP_V2) and snp_01_0005 (195,557,990 bp AGP_V2). Positions are based on B73 genome sequence release AGP_V2 (Schnable *et al.,* 2009). An additional 874 individual F_3 plants were identified as recombinants from the F_3 population. Recombinant plants were self-pollinated and homozygous recombinants identified. Homozygous recombinants were increased and evaluated for disease resistance. Subsequently, a population of 1,546 F_3 plants was screened from snp_01_0059 (184,633,349 bp AGP_V2) to snp_01_0083 (189,352,206 bp AGP_V2), yielding an

additional 156 recombinants. Homozygous plants were identified, self-pollinated and screened for NLB and Stewart's wilt (Fig.1).

Disease trials

Northern leaf blight

NLB trials were carried out at the Cornell University Robert Musgrave Research Farm in Aurora, NY. In the fine-mapping populations, 194, 80, and 146 homozygous 'fixed' recombinants were screened for NLB in 2010, 2011, and 2012, respectively (Fig. 1). Lines carrying mutations in the genes *pan1* and *pan2* were tested in Aurora, NY in 2011, 2012, and 2013, and in Ithaca, NY in 2011. Plants were inoculated with *S. turcica* isolate StNY001 (race 1) using inoculation procedures described previously (Chung *et al.,* 2010). Briefly, cultures of the fungus were grown on lactose casein agar for three to four weeks prior to inoculation or sorghum culturing. A spore suspension was prepared by flooding the cultures with 5 mL sterilized distilled water and conidia were dislodged using a glass rod. The spore suspension was filtered through two layers of cheesecloth and adjusted to a concentration of $4x10^3$ spores per mL using a haemocytometer. The spore suspension contained a final concentration of 0.02% Tween 20. Sorghum seed cultures were prepared by soaking 900 mL of sorghum grains in 600 mL distilled water overnight in a one-gallon clear milk jug and autoclaved twice for 25 minutes. One mL of unfiltered spore suspension was then introduced to each jug, which was then cultured at room temperature for about three weeks before field inoculations were conducted. Jugs were shaken daily to prevent caking and provide uniform infestation. For field inoculations, 0.50 mL spore

suspension and ~1.25 mL sorghum grains colonized by *S. turcica* were placed into the whorl of each plant at the five- to six-leaf stage.

Diseased leaf area (DLA) was rated on a per row basis three times after flowering at an interval of seven to ten days using a percentage scale of 0-100 with increments of one, where 0 indicates a plant with no disease and 100 indicates a completely diseased plant. Area under the disease progress curve (AUDPC) was calculated as described previously (Chung *et al.,* 2010).

Stewart's wilt

Stewart's wilt trials were conducted at the Musgrave Research Farm in Aurora, NY in 2010, 2011, and 2012 for fine-mapping and 2012 and 2013 for *pan1* and *pan2* mutants. Due to flooding in 2013, it was not included in the analysis. Plants were inoculated with *Pantoea stewartii* strain PsNY003, originally collected in NY in 1991, at the five- to six-leaf stage, with inoculum prepared and a modified pinprick method used for inoculations as described previously (Chung *et al.,* 2010). DLA was rated on a per row basis at two to four weeks after inoculation using a percentage scale of 0 to 100, with 0 being no disease and 100 being completely diseased.

Genotyping assays

SNP marker development

The maize diversity project database http://www.panzea.org (Canaran *et al.,* 2008) was used to locate polymorphisms between the two inbred lines using a number of datasets including markers from the NAM genetic map (McMullen *et al.,* 2009),

HapMapV1 (Gore *et al.,* 2009), and HapMapV2 (Chia *et al.,* 2012). Single nucleotide polymorphisms (SNPs) polymorphic between B73 and Tx303 were chosen for this study. SNP markers used for the fine-mapping study are shown in Table 1. In addition, an Illumina MaizeSNP50 Beadchip assay (Illumina, San Diego, CA, USA) was conducted on pooled DNA from families TBBC3-38_05F and TBBC3-38_19E at the David H. Murdock Research Institute, (Kannapolis, NC, USA) which included genotypic information for 52,686 SNPs.

DNA extractions

Both Sigma ExNAmp (Sigma-Aldrich, St. Louis, MO, USA) and CTAB DNA extractions were used. ExNAmp DNA extractions were used to identify recombinants and homozygous 'fixed' recombinants. CTAB extractions were used for genotype confirmation and breakpoint analysis. To perform ExNAmp extractions, 1 mm^2 of plant tissue was collected in a 0.2-mL PCR tube and the tubes were placed on ice. Extraction buffer (8 µL) was added to each tube, and tubes were incubated at 95°C for ten minutes. Following the incubation, 8 µL of dilution buffer was added. The resulting DNA was diluted 1:100 with water for KASPar genotyping. CTAB DNA extractions were performed using about 0.1 mg of fresh tissue as described previously (Doyle and Dickson 1987, Chung *et al.,* 2010).

Allele-specific PCR

When using ExNAmp-extracted DNA, 10 µL of 1:100 diluted DNA was dried down in a 384-well KASPar plate (LGC Genomics, Hoddesdon, Hertfordshire, UK) and a 4-

μL reaction performed. For reactions using CTAB extracted DNA, DNA was quantified and approximately 5 ng/μL of DNA was used per reaction. Reaction conditions were as follows: 1x KASPar reaction mix (LGC Genomics, Hoddesdon, Hertfordshire, UK), 0.4 mM $MgCl_2$, 0.41 mM common reverse primer, and 0.165 μM of each allele-specific primer. Standard oligonucleotides were obtained from IDT (Coralville, IA, USA). PCR thermocycling parameters were as follows: 94°C for 15 minutes, 20 cycles of 94°C for 10 seconds, 57°C for 5 seconds, and 72°C for 10 seconds, followed by 26 cycles of 94°C for 10 seconds, 57°C for 20 seconds, and 72°C for 40 seconds. Results were read using an Applied Biosystems 7900 HT (Life Technologies, Grand Island, NY, USA) and analyzed using SDS v2.1 (Life Technologies, Grand Island, NY, USA).

*Experimental design and statistical an*al*ysis*

A randomized incomplete block design was used for all fine-mapping field experiments, with three replications for NLB trials and two for Stewart's wilt trials. NILs carrying B73 and Tx303 alleles across the region were included in each block as check lines. Two rows were planted around the edge of the experiment to reduce border effects. For the breakpoint analysis, best linear unbiased predictors (BLUPs) were calculated using the 'lmer' command in the lme4 package in R version 2.14 (R Core Development Team 2013) where line, year, replication within year, and block nested within replication were fitted as random factors in a mixed-effects model for NLB. Similarly, Stewart's wilt BLUPs were calculated using the 'lmer' command including line and year as random effects. Fine-mapping statistical analyses were

completed in R version 2.14 (R Core Development Team 2013) using R/qtl (Broman *et al.,* 2003). First, individuals with fewer than 11 genotyped markers and markers with fewer than 250 individuals genotyped were removed from the analysis. A genetic map was then constructed using the Kosambi mapping function (Kosambi 1943). Single marker regression was conducted (Paterson *et al.,* 1990, Kump *et al.,* 2010) using the 'scanone' function in R/qtl. Confidence intervals were calculated on the basis of a 95% Bayes credible interval using the function 'bayesint' in R/qtl (Broman *et al.,* 2003).

Recombination rate diversity

Recombination rates were calculated using genotyping-by-sequencing SNPs on the NAM sub-populations, using phased and fully imputed at 1-cM resolution genotypes (AllZea_GBSv2.3) (http://panzea.org/lit/data_sets.html). Genetic maps were constructed using R/qtl with the "est.map" function in R (Broman *et al.,* 2003, R Core Development Team 2013).

Read depth variation

HapMapV2 aligned sequencing reads for B73 and Tx303 (Chia *et al.,* 2012) were downloaded from iPlant (Goff *et al.,* 2011). SAMtools was used to count the number of reads at each nucleotide location (Li *et al.,* 2009). A Perl script was written to divide the interval into 11 bins of equal size (327,181 bp) and to compile the number of reads per bin.

Candidate gene identification

All B73 RefGenV2 (AGP_V2) filtered genes between the two flanking markers snp_01_0047 at 185,737,089 bp (AGP_V2) and snp_01_0082 at 189,336,643 bp (AGP_V2) of the narrowed NLB fine-mapping interval were considered as candidate genes. SNPs from genome-wide nested association mapping (Poland *et al.,* 2011, Chia *et al.,* 2012) with a bootstrap posterior probability (BPP) >0.01 that fell within the narrowed fine-mapping interval were considered further as candidates.

Association analysis of the 282- line maize diversity panel

Association mapping was conducted for the NLB fine-mapping interval. BLUPs that included design factors, flowering time, and population structure were used for association analysis (Wisser *et al.,* 2011). A mixed linear model (MLM) was implemented using TASSEL v4 (Bradbury *et al.,* 2007). The markers assayed included 47,445 Illumina MaizeSNP50 SNPs (Cook *et al.,* 2012) and 425,035 genotyping-by-sequencing SNPs (Romay *et al.,* 2013), filtered to remove sites with >20% missing data (Olukolu *et al.,* 2013). The kinship (K) matrix was constructed in TASSEL using a 5,000-SNP subset of the Illumina MaizeSNP50 dataset (Cook *et al.,* 2012) that had no missing data (Olukolu *et al.,* 2013). Q values were calculated using qvalue package (Storey 2002). Associations with a false discovery rate of FDR<0.15 were noted.

Mutant analysis

Mutants in the *pan1* and *pan2* genes were evaluated for NLB and Stewart's wilt

reaction. Two mutant alleles of *pan1* were evaluated in a B73 background: one mutant generated by ethyl methanesulfonate mutagenesis (*pan1-EMS*) and one line with a *Mutator1* (*Mu1*) transposon in *pan1* (Gallagher and Smith 2000, Cartwright *et al.,* 2009). Two ethyl methanesulfonate alleles of *pan2* were evaluated in a B73 background: *pan2-0* and *pan2-3* (Zhang *et al.,* 2012). All *pan1* and *pan2* mutant alleles except *pan2-O* can be considered null alleles based on the nature of the mutations and analysis of PAN protein accumulation in mutants, whereas *pan2-O* is a missense allele that may encode a partially functional protein (Zhang *et al.,* 2012). For mutant analysis a complete block design was used, with five replications per location for *pan1* and *pan2* mutants. NLB results were analyzed with a mixed linear model in JMP 9.0 (SAS, Cary, NC, USA), with genotype as a fixed effect and replication nested within environment, and environment as random effects. Stewart's wilt results were analyzed with genotype as a fixed effect and replication as a random effect.

RT-PCR analysis

Tissue for RNA extraction was collected from mature leaf tissue of $(BC_4F_3)BC_1F_5$ plants carrying either the B73 ($qNLB1.06_{B73}$) or Tx303 allele ($qNLB1.06_{Tx303}$) at the *qNLB1.06* locus during the summer of 2011 and 2012. RNA was extracted using an RNeasy kit (Qiagen) and first cDNA was prepared from this RNA using a RETROscript First Strand cDNA Synthesis kit (Life Technologies). PCR was carried out using the following primers for amplification of *pan1* (5'-TCGGGATGGAGCTGGAGGAG-3' and 5'-TGGACAGACGCACGGACCAC-3') and actin as a control (5'-TCAGCAGGTCTTCTCTTTCTT-3' and 5'-

TCCTTCATATTTCCTTCGTTC-3') and Q5 Hot Start Taq Polymerase (New England Biolabs). *pan1* and actin PCR products were quantified from gel images using NIH ImageJ vs. 1.47g.

Results

Identification of multiple disease resistance in bin 1.06

A number of QTL studies have localized resistance to NLB to maize bin 1.06, with varying resolution (Table 2). These studies have consistently implicated the region spanning from 180 to 205 Mb. Lines carrying a Tx303 introgression at this interval were found to be associated with resistance to NLB and Stewart's wilt (families TBBC3-38 and TBBC-39 of the TBBC3 population (Chung *et al.,* 2010). Based on genetic background and seed availability, TBBC3-38_19E, TBBC3-38_15A, and TBBC3-38_17G were selected for fine-mapping. The Tx303 introgression in TBBC3-38 spans from ss196428597 (172,877,033 bp) to ss196518155 (196,244,799 bp) (Fig. 2). An interval of 15.16 Mb spanning from snp_01_0042 (180,394,890 bp AGP_V2) to snp_01_0005 (195,557,990 bp AGP_V2) was targeted for fine-mapping, based on NIL introgression locations and previous QTL mapping studies (Table 2 and Fig. 2).

Fine-mapping of multiple disease resistance at 1.06

Two markers flanking the *qNLB1.06* region, snp_01_0042 and snp_01_0005, were used to screen 435 F_2 and 4,475 F_3 plants. We identified a total of 1,130 recombinants spanning the 15 Mb interval of interest. Plants were self-pollinated and progeny were assayed for homozygous recombinants. Recombinant plants were selected for

phenotypic evaluation based on breakpoint analysis and seed availability. Fixed recombinants were screened for disease phenotype in a randomized incomplete block design with two control NILs derived from a single F_4 individual, one carrying the B73 allele and one carrying the Tx303 allele in the region of interest. Fixed recombinants were screened for NLB (n=194, 80, and 146 in 2010, 2011, and 2012, respectively) and Stewart's wilt (n=60, 78, and 140 in 2010, 2011 and 2012, respectively) (Fig. 2). An additional 17 SNP markers were assayed on the population to determine the physical position of breakpoints (Table 1). Five were removed from the breakpoint analysis because of missing genotypes or a lack of recombination with neighboring markers. The order of the physical map matched the order of the genetic map. Based on these data, *qNLB1.06* was narrowed to a 3.60-Mb region flanked by snp_01_0047 (185,737,089 bp AGP_V2) and snp_01_0082 (189,336,643 bp AGP_V2) (Fig. 2). *qSw1.06* was narrowed to a 95.9-kb interval flanked by snp_01_0137 (187,245,104 bp AGP_V2) and snp_01_0139 (187,341,010 bp AGP_V2) (Fig. 2). While confidence intervals differed for the diseases, breakpoint analyses for both showed similar profiles. The smaller confidence interval for Stewart's wilt may reflect the stronger phenotype, however, the similar profile indicates there may be multiple genes underlying the QTL for both diseases.

Genomic integrity

We observed a low frequency of recombination across part of the fine-mapping region. To determine whether this was an anomaly only found in the NIL fine-mapping population, we examined the recombination rates across the RIL populations

that comprise the NAM population by examining genetic distances in NAM sub-populations, as shown in Fig. 3. We found a depressed recombination rate across portions of the target interval in the Tx303 x B73 RIL population, confirming a low rate of recombination found in the current study between B73 and Tx303 in this region. Furthermore, most NAM founder lines showed low recombination rates across parts of the region. Hp301, however, showed higher levels of recombination, suggesting structural similarity between B73 and Hp301. Other lines, such as Ms71 and M37W, showed repressed recombination in other regions of the interval. Because low recombination rates are hypothesized to be due to structural variation such as inversions, indels, transposable elements, or presence/absence variation (McMullen *et al.,* 2009), we examined read depth variation across the interval in an attempt to identify structural variation. We found variation in the number of Tx303 reads that mapped to the B73 reference sequence, while little variation was observed in the number of B73 reads that mapped to the reference sequence (Fig. 4). The region with little to no recombination in the NAM subpopulations showed a reduced number of reads in Tx303. Conversely, the region with significant NAM GWAS associations had an elevated number of reads that mapped to the reference genome in this location, suggesting possible duplications and genome expansion.

Candidate genes underlying $qSw1.06_{Tx303}$

The Stewart's wilt fine-mapping region was narrowed to a 95.9-kb interval that contains a putative zinc finger (GRMZM2G445684) and two uncharacterized genes: GRMZM2G445676, and AC213857.4_FG001. The two uncharacterized genes have

no known homologs and no annotated domains. Resistance at this locus is conferred by Tx303, and it is therefore plausible that the resistance gene is absent from the B73 reference.

Candidate genes underlying qNLB1.06$_{Tx303}$

The fine-mapping region falling within the Bayes 95% confidence interval for NLB resistance, 185.7 Mb to 189.3 Mb of maize chromosome 1, comprises 117 annotated coding genes, 30 pseudogenes, and 39 transposable elements in the B73 genome sequence (Table 3). A number of genes within the narrowed fine-mapping interval are credible candidates based on the involvement of those gene classes in plant defense as documented in the scientific literature. These include three putative leucine rich repeat-encoding genes, three putative protein kinases, two putative wall-associated receptor kinases, and one putative lipoxygenase sharing homology with *Arabidopsis thaliana LOX2*. Furthermore, genome-wide nested association mapping conducted by Chia *et al.,* (2012) identified three significant associations clustered within the 40-kb region spanning 187.23 to 187.27 Mb AGP_V1. The most significant hit within the region was an intergenic 10 kb read depth variation with a bootstrap posterior probability (BPP) of 44, which was 30 kb upstream of a putative serine-threonine protein kinase and 129 kb downstream of a putative zinc-finger encoding gene (GRMZM2G441903). Two additional polymorphisms, one intronic SNP (BPP=3) and one intergenic copy number variation (BPP=1) were within 40 kb of the significant association with BPP=44.

Association mapping

Association analysis of this region using the 282-line Goodman diversity panel (Flint-Garcia *et al.,* 2005) identified an association between 185.7 Mb to 189.3 Mb (Fig. 5). For significant intergenic associations, adjacent genes were considered as well as genes implicated by long-distance linkage disequilibrium. The SNP implicated by association analysis was located at 188,018,070 bp (AGP_V2) (p-value=1.72×10^{-4}, q-value=0.136), 260 bp downstream of a SpoU methylase (GRMZM5G854901) and 13.54 kb upstream of an uncharacterized gene with a helix-loop-helix DNA-binding domain (GRMZM5G879527). The receptor-like kinase *pan1* is located 37.555 kb from the significant diversity panel association, a distance close enough to link the SNP to *pan1* by long-range LD (Chia *et al.,* 2012) or the SNP could implicate a downstream element regulating the expression of *pan1*.

pan1 *is a susceptibility gene for NLB*

pan1 was initially a candidate gene for disease resistance at 1.06 based on its location inside the fine-mapping interval, its proximity to an NLB association from the diversity panel analysis, and its identity as a receptor-like kinase (Cartwright *et al.,* 2009) (this class of proteins is known to detect microbe- associated molecular patterns; (Zipfel 2008)). Phenotypically similar, but unlinked, *pan2* mutants (Zhang *et al.,* 2012) were also assessed to test the hypothesis that the *pan* genes influence the disease response through their known effect on stomatal morphology or related pathways. We tested *pan1* and *pan2* mutants in replicated, multi-year trials and found a significant genotype effect (p-value <0.0001). Both mutants with null alleles of *pan1*

were found to be significantly more resistant to NLB than B73, indicating *pan1* is a susceptibility gene for NLB, while *pan2* mutants showed no significant difference from B73 (Fig. 6). For NLB, *pan1-Mu* had a 41% decrease in AUDPC, as compared with B73, and was generally more resistant than *pan1-ems*, which had a 27% decrease in AUDPC, as compared to B73. For Stewart's wilt, pan1 mutants were nearly immune.

These results suggest that partial or complete loss of *pan1* may contribute to the increased resistance seen in $qNLB1.06_{Tx303}$ compared to $qNLB1.06_{B73}$. To further test this hypothesis, we compared *pan1* gene expression levels in mature leaves of these two lines via RT-PCR. Consistent with reduced *pan1* function in $qNLB1.06_{Tx303}$, we found that *pan1* to control actin signal ratios were decreased from 0.915 +/- 0.168 in $qNLB1.06_{B73}$ to 0.553 +/- 0.035 in $qNLB1.06_{Tx303}$ (+/- standard errors, $p<0.05$ using Student's T test). However, further work will be needed to determine whether the reduction in *pan1* expression level in $qNLB1.06_{Tx303}$ is causally related to the increase in NLB resistance seen in this line.

Discussion

Loci that underlie a number of traits present an opportunity to investigate the complex relationship between variation for traits, genome structure, recombination, and causative genes. Maize bin 1.06 is one such locus that is associated with effects on diverse traits. This chromosomal region of interest has been described as a yield-stabilizing locus associated with effects on resistance to several diseases, root architecture, plant height, flowering time and yield across different soil moisture levels

and genetic backgrounds (Landi *et al.,* 2002, Tuberosa *et al.,* 2002, Wisser *et al.,* 2006, Landi *et al.,* 2010). This locus is a relatively QTL-dense segment of the maize genome, with more than double the average number of QTL (35 QTL v. an average of 15 QTL/bin), but an average genetic size on the NAM genetic map and physical size (McMullen *et al.,* 2009, Andorf *et al.,* 2010). The elevated number of QTL in this bin, coupled with an average gene content and genetic size, indicate that this bin is important for maize breeding across a broad set of traits. Hence, it is of interest not only to identify the genes underlying those traits, including multiple disease resistance, but also to investigate the genome dynamics shaping the region.

Consistent with the observation of high QTL density, variations in the targeted interval were shown to be under selection during domestication and subsequent varietal improvement. Candidate genes for domestication syndrome in this region, identified as genes lying in extended regions with allele frequency differentiation between landraces and *Zea mays* ssp. *parviglumis*, include genes such as a putative lipoxygenase, putative frataxin, and a putative zinc finger among others, while "improvement candidates" (those that contrast for improved lines versus landraces) include putative protein kinases, putative EF-hand proteins, a putative alcohol dehydrogenase, and a putative antifreeze protein, among others (Hufford *et al.,* 2012). The observed patterns of selection may be related to disease resistance, as the transcriptional rewiring of the maize transcriptome during domestication suggests that genes related to biotic stress are overrepresented among the group of genes up-regulated during domestication (Swanson-Wagner *et al.,* 2012). Indeed, some of these candidate genes, such as the lipoxygenase, serine/threonine protein kinase, and the

antifreeze protein, could be involved in one of the many disease resistances conditioned by this locus.

This region harboring numerous QTL for diverse traits shows signs of high genome complexity and plasticity. A low recombination rate in the fine-mapping interval was observed in the NILs, with a limited number of recombinants identified between 184.6 Mb and 187.6 Mb, but an average gene density as compared to the maize genome as a whole (Schnable *et al.,* 2009). The fine-mapping interval, including the region of low recombination, co-localizes with the yield-stabilizing QTL reported by Landi *et al.,* (2010). A reduced recombination rate in this interval was observed in many of the NAM subpopulations, providing support for the hypothesis that B73 has a lack of synteny with other maize lines at this region, with the exception of Hp301, which recombines with B73 in this region. The low recombination could be due to small inversions, indels, transposon insertion, or presence/absence variation (McMullen *et al.,* 2009). Such differences that suppress recombination may be selected upon to conserve the yield-stabilizing haplotype located at this region.

Increasingly, copy number variation (CNV) has been found to underlie trait variation, including biotic and abiotic stress tolerance (Cook *et al.,* 2012, Maron *et al.,* 2013). A CNV polymorphism was significantly associated with NLB in the 1.06 interval based on the NAM GWAS. Together with the lack of recombination in part of the fine-mapping population, this suggests that genome content variation across diverse maize germplasm may underlie the differences in disease response. To test this hypothesis, structural differences were explored by examining read depth variation across the region. Pronounced variation in the number of Tx303 reads that mapped to

the reference was found, which can be interpreted as evidence for duplications or genome expansion at this region in Tx303. This is not uncommon, as the maize genome is highly plastic, with read depth variation in 90% of the genome (Chia *et al.,* 2012) and presence/absence variation thought to be a major driver of phenotypic variation in maize (Wallace *et al.,* 2014).

Given the complex genetic basis of quantitative traits and this region, a fine-mapping approach was taken to refine the genomic region associated with resistance to NLB and Stewart's wilt, complemented by association mapping to identify candidate genes. The fine-mapping approach allowed for the dissection of the multi-trait nature of this QTL. Resistance to Stewart's wilt was localized to a 95.9-kb region within the larger 3.60 Mb NLB fine-mapping interval. While the majority of major QTL have been shown not to be pleiotropic in nature (Wallace *et al.,* 2014), a pleiotropic basis of disease resistance cannot be excluded at this locus. The breakpoint analysis for both diseases was similar, although the confidence interval of NLB was calculated to be larger than that for Stewart's wilt.

The candidate region for Stewart's wilt contains three genes in the B73 genome sequence: a gene with a putative zinc finger and two uncharacterized genes. Both uncharacterized genes lack homologs and one lacks expression evidence (Dong *et al.,* 2004, Sen *et al.,* 2010). However, the genic content of the region may differ in Tx303. A physical map assembly for Tx303 across the fine-mapping region would clarify this. A number of mapping studies have implicated the region on Chr. 1 between 180 and 190 Mb across diverse populations for resistance to NLB. The $qNLB1.06_{Tx303}$ region has been successfully narrowed to 3.6 Mb. While 117 candidate

genes from the B73 genome sequence were implicated through fine-mapping, it is likely that Tx303 differs in the genic content of this region. A subset of the 117 genes was particularly credible based on previous knowledge of plant defense, including four putative leucine-rich repeat protein kinases. Other candidates include a frataxin, an ABC transporter, and a lipoxygenase.

Association mapping provides a complementary approach for identifying candidate genes. Significant associations within the *qNLB1.06* fine-mapping interval were detected using both the NAM and Goodman diversity panels (Flint-Garcia *et al.,* 2005, Poland *et al.,* 2011, Wisser *et al.,* 2011, Chia *et al.,* 2012). The most significant association within this region in the NAM was an intergenic copy number variation, which had a BPP value of 44 (*p*-value= 0.0000737), one of the most highly significant associations from the analysis (Poland *et al.,* 2011, Chia *et al.,* 2012). Among the genes implicated by NAM, the putative A20/AN1 zinc finger was the strongest candidate; a gene domain that is associated with stress tolerance in plants and the immune system in animals (Vij and Tyagi 2008). This polymorphism is close to the Stewart's wilt fine-mapping interval, lending support to the hypothesis that a region present in Tx303 but not B73 may contain gene(s) for resistance to both diseases. Association analysis using the Goodman diversity panel revealed a significant intergenic SNP within the fine-mapping interval, approximately 800 kb from the NAM CNV (Flint-Garcia *et al.,* 2005, Wisser *et al.,* 2011). The NLB-associated CNV and SNP polymorphisms may be in linkage disequilibrium with one or more genes in the vicinity. Candidate genes from association mapping can be further investigated through expression analyses, re-sequencing, and testing across different germplasm

sets.

Within the fine-mapping interval shown in Fig. 2, the significant Goodman panel SNP was 38 kb from *pan1*, as shown in Fig. 5. We tested the *pan* mutants for both NLB and Stewart's wilt because of the similar mapping results between the two diseases (Fig. 2). The smaller confidence interval for Stewart's wilt may reflect the stronger phenotype, however, the similar profile indicates there may be multiple genes underlying the QTL for both diseases. Two independent null mutant alleles of this gene conferred resistance phenotypes for NLB and Stewart's wilt (Fig. 6), demonstrating that mutations in *pan1* itself (not a linked gene present in one or other mutant background) increase resistance for the two diseases. This finding suggests that a loss of function allele of *pan1* derived from Tx303 may contribute to the disease resistance phenotype(s) conferred by $qNLB1.06_{Tx303}$ and $qSW1.06_{Tx303}$; further work is needed to test this hypothesis directly. *pan1* has been studied for its role in promoting features of actin organization that support asymmetric cell division (Cartwright *et al.,* 2009). Interestingly, lines carrying mutations for *pan2*, also a gene studied for its role in asymmetric cell division, were not significantly different than B73, indicating aberrant stomata found in both mutant lines are not the underlying mechanism of resistance in the *pan1* mutants. Susceptibility conditioned by wild-type *pan1* could be due to a passive mechanism, such as altered anatomical structures, or an active process, such as actin re-organization during pathogen attack.

We have successfully refined *q1.06Tx303*, identified candidate genes, and demonstrated a role for *pan1* in multiple disease resistance. NLB and Stewart's wilt resistance regions have been narrowed sufficiently that the markers within the NLB

and Stewart's wilt intervals can be used for marker-assisted selection. These data strongly suggest that structural variation underlies this locus and *pan1*, a gene in which lowered expression is correlated with higher resistance levels, may underlie the NLB QTL. Loss of susceptible *pan1* alleles could be used to decrease maize susceptibility to diverse pathogens. Through fine-mapping, examining recombination rates and re-sequencing data, and evaluating mutant lines, we were able to dissect a complex locus and identified a role for *pan1* in plant defense. This approach shed light on a locus known for its complexity and quantitative effect.

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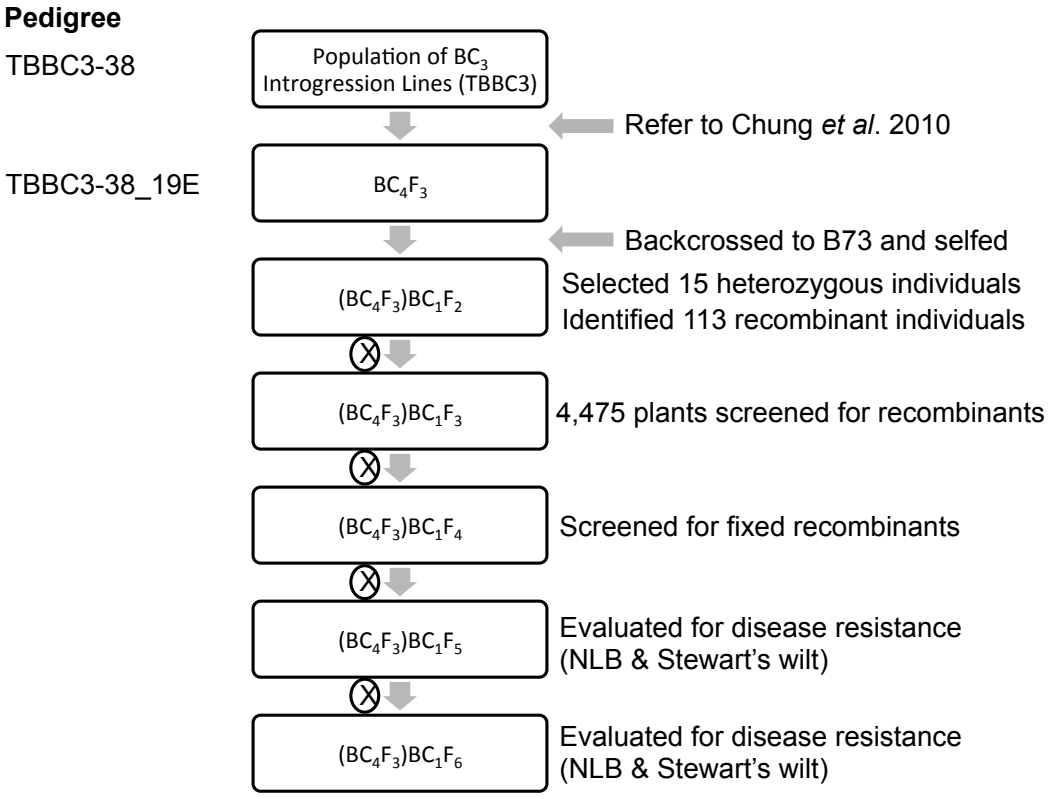

Figure 1: Near-isogenic line development. Near-isogenic line development is shown beginning with population development by Szalma *et al.* (2007) and Chung *et al.* (2010), through fixed recombinant screening.

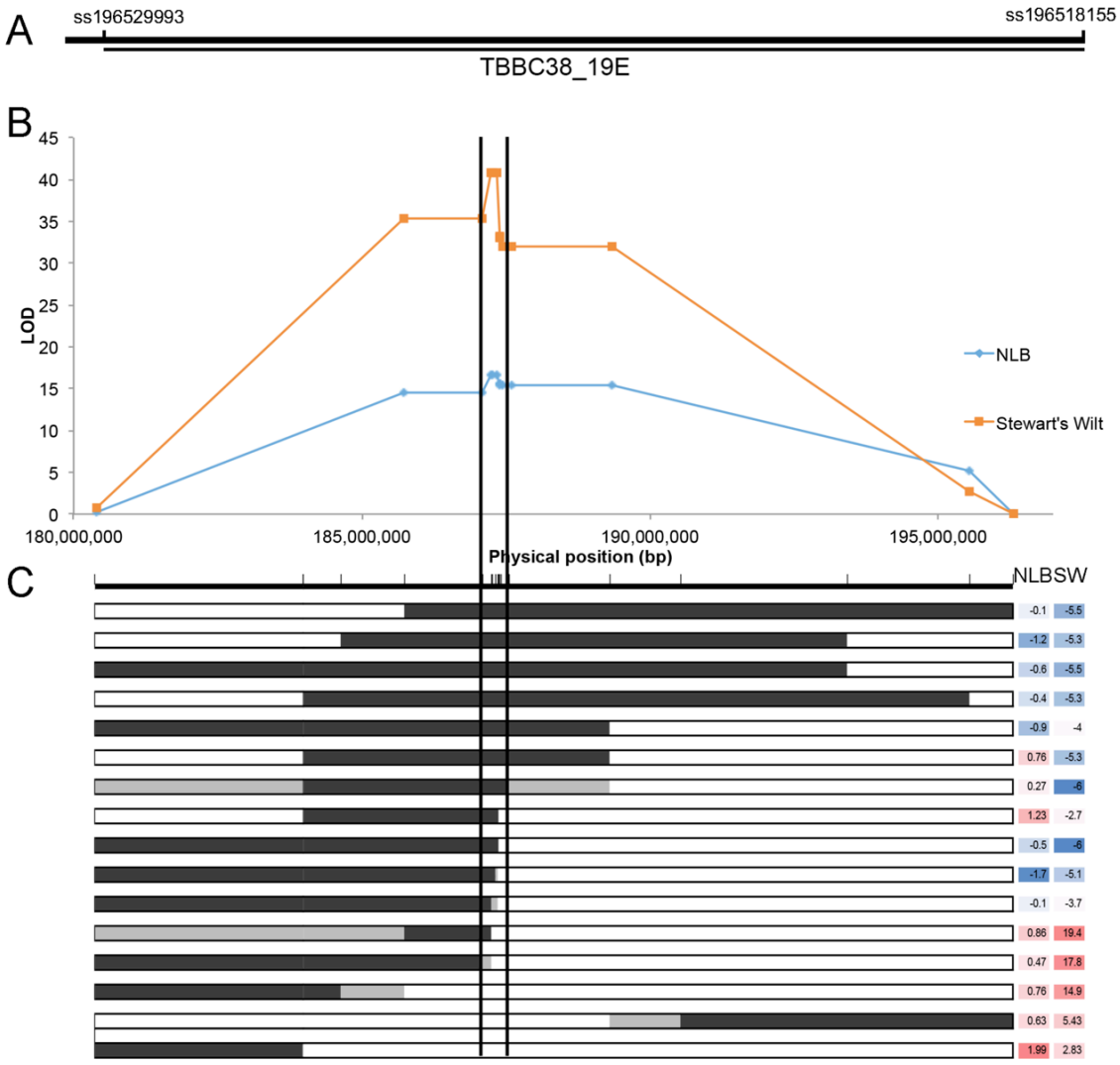

Figure 2. Mapping of *qNLB1.06* and breakpoint analyses for *qNLB1.06.*

A.) Locations of introgressions for NILs TBBC3_5F and TBBC3_19E are shown with marker names. B.) Breakpoint analysis for NLB and Stewart's wilt. C.) Selected representative recombinants and their associated phenotypes. For genotypes, dark shading indicates the Tx303 allele, while white indicates the B73 allele. Light grey shading indicates the region of a recombination event. For phenotypes, blue shading indicates a more resistant line, while red indicates a more susceptible line.

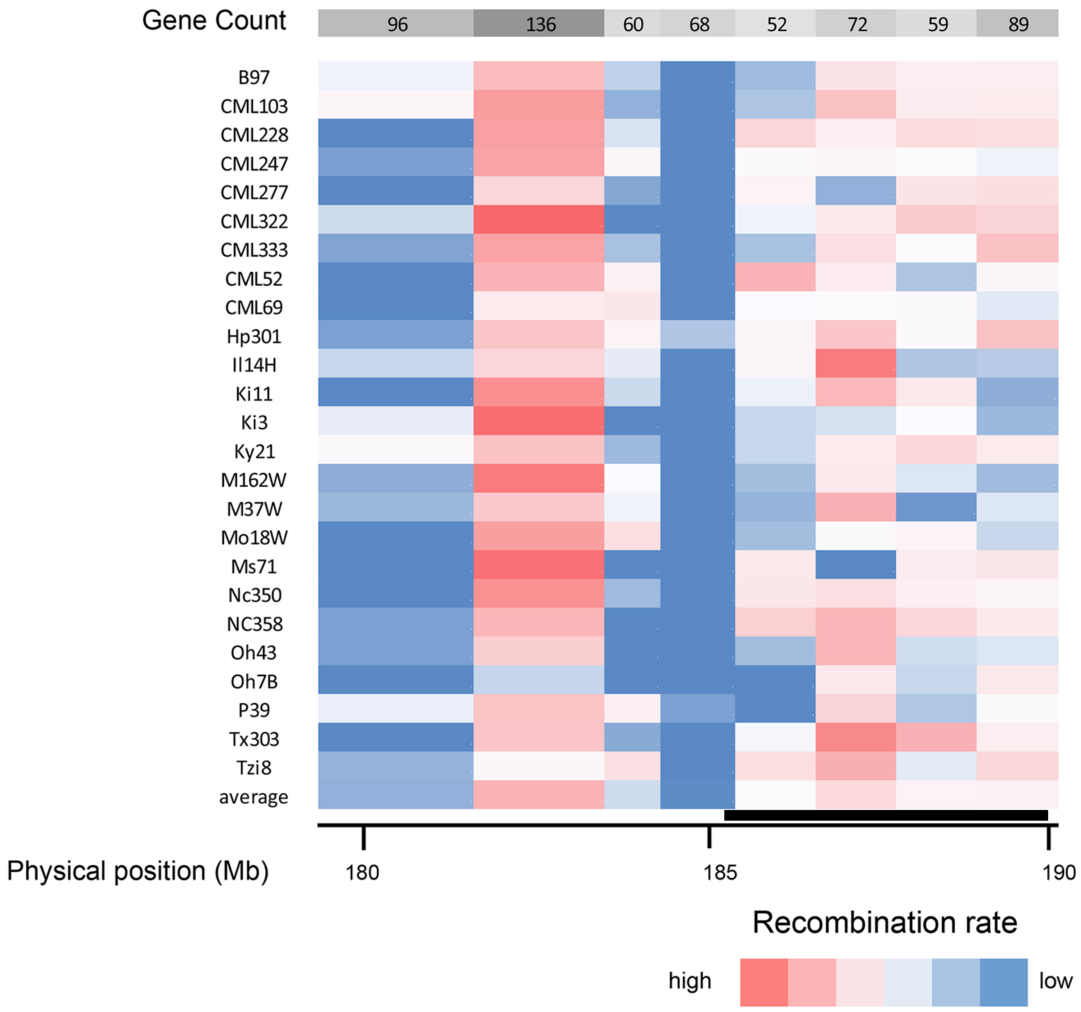

Figure 3. Recombination rates. Recombination rates were calculated between nine markers for the NAM sub-populations. NAM founders are shown on the left and the gene counts for the eight sub-intervals across the top. Red indicates regions of high recombination and blue indicates regions of low recombination. The black bar above the physical positions represents the region examined for read depth variation in Figure 4.

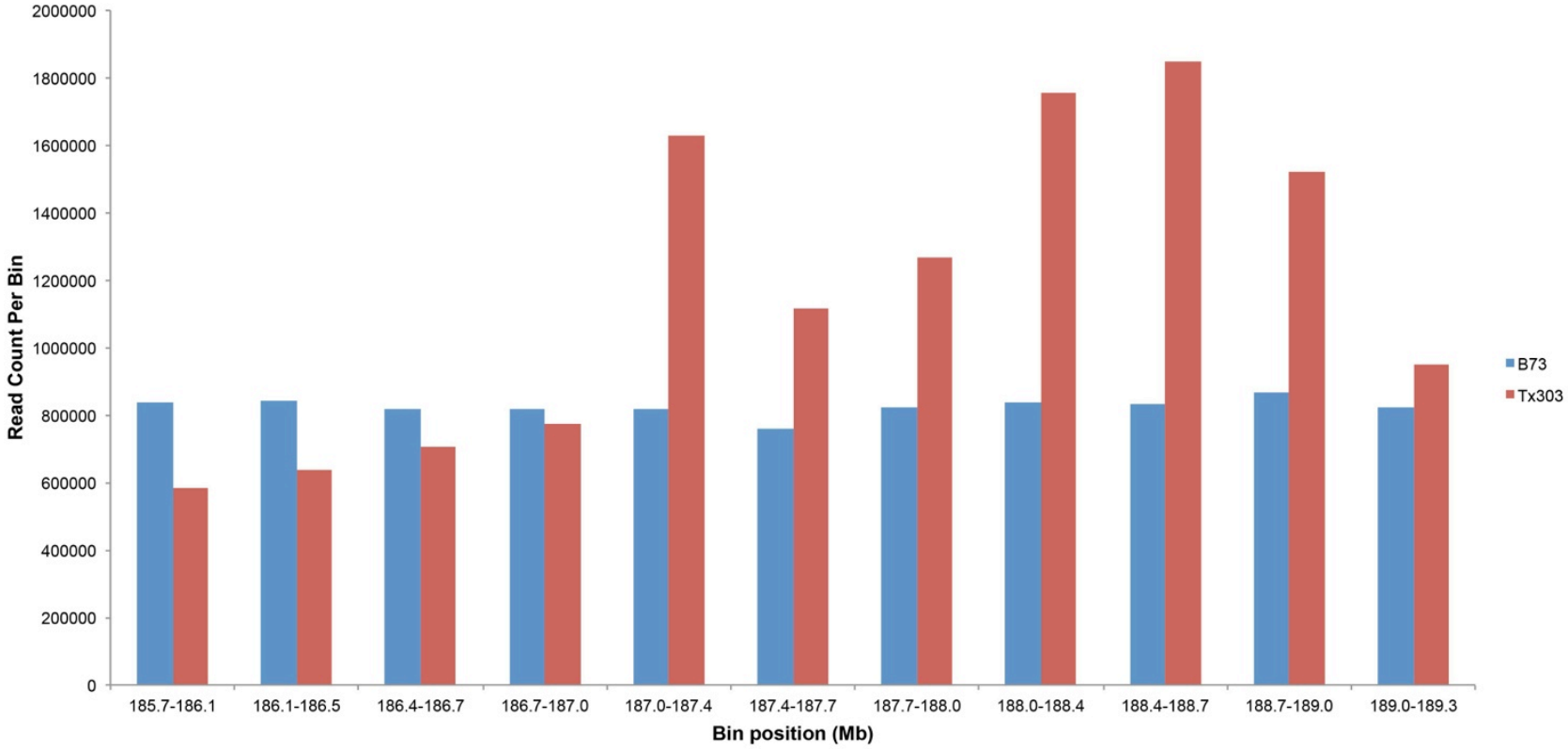

Figure 4. Read depth variation across *qNLB1.06* fine-mapping region.

Blue bars indicate the number of B73 Illumina reads that align to the given bin, while red bars indicate the number of Tx303 Illumina reads that align to the given bin. Bins are 327 kb and the positions of the bins in noted on the X-axis.

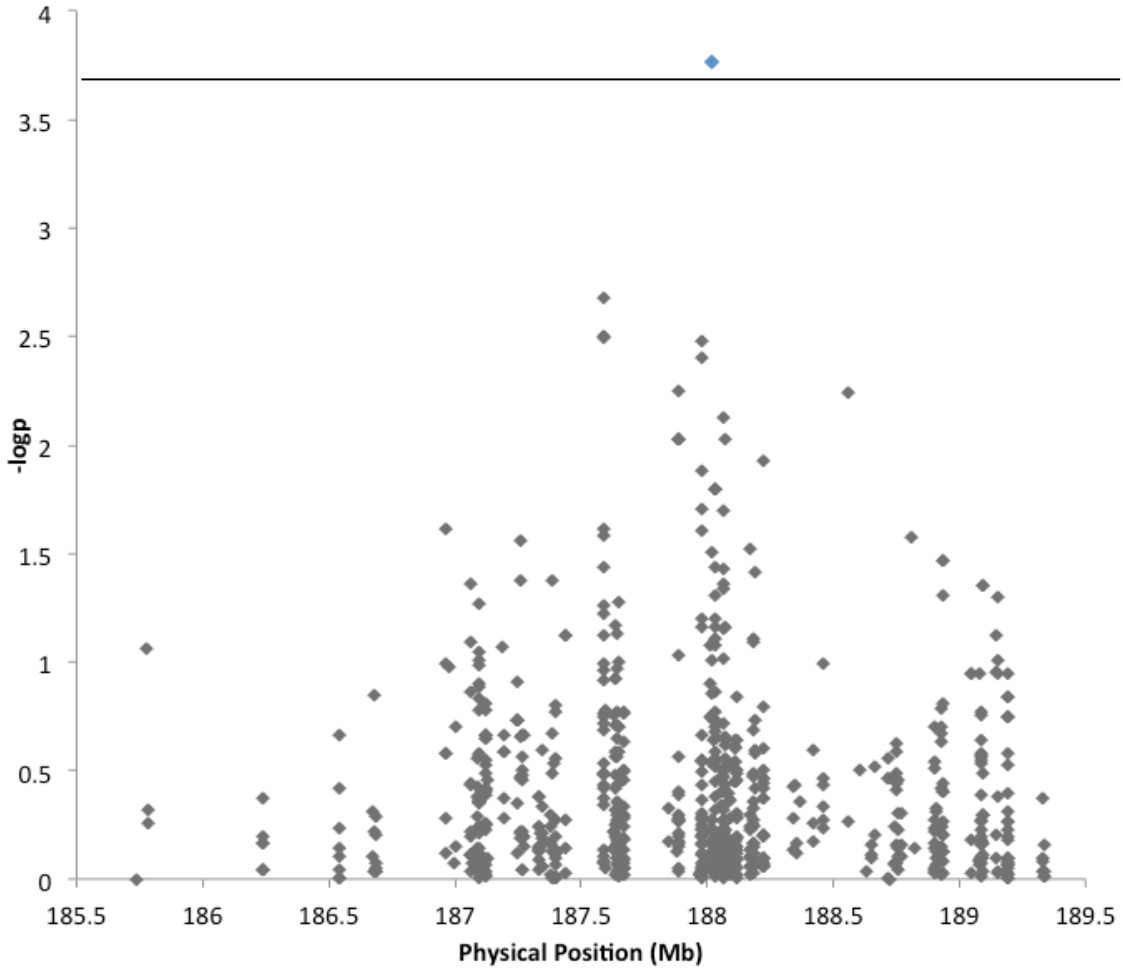

Figure 5. Diversity panel association. Association analysis for NLB in the fine-mapping region using the Goodman diversity panel (Flint-Garcia *et al.,* 2005). The significant SNP at 188,018,070 bp (*p*-value=1.72x10-4, q-value=0.136) is blue. The false discovery rate threshold is represented by a q-value of 0.15.

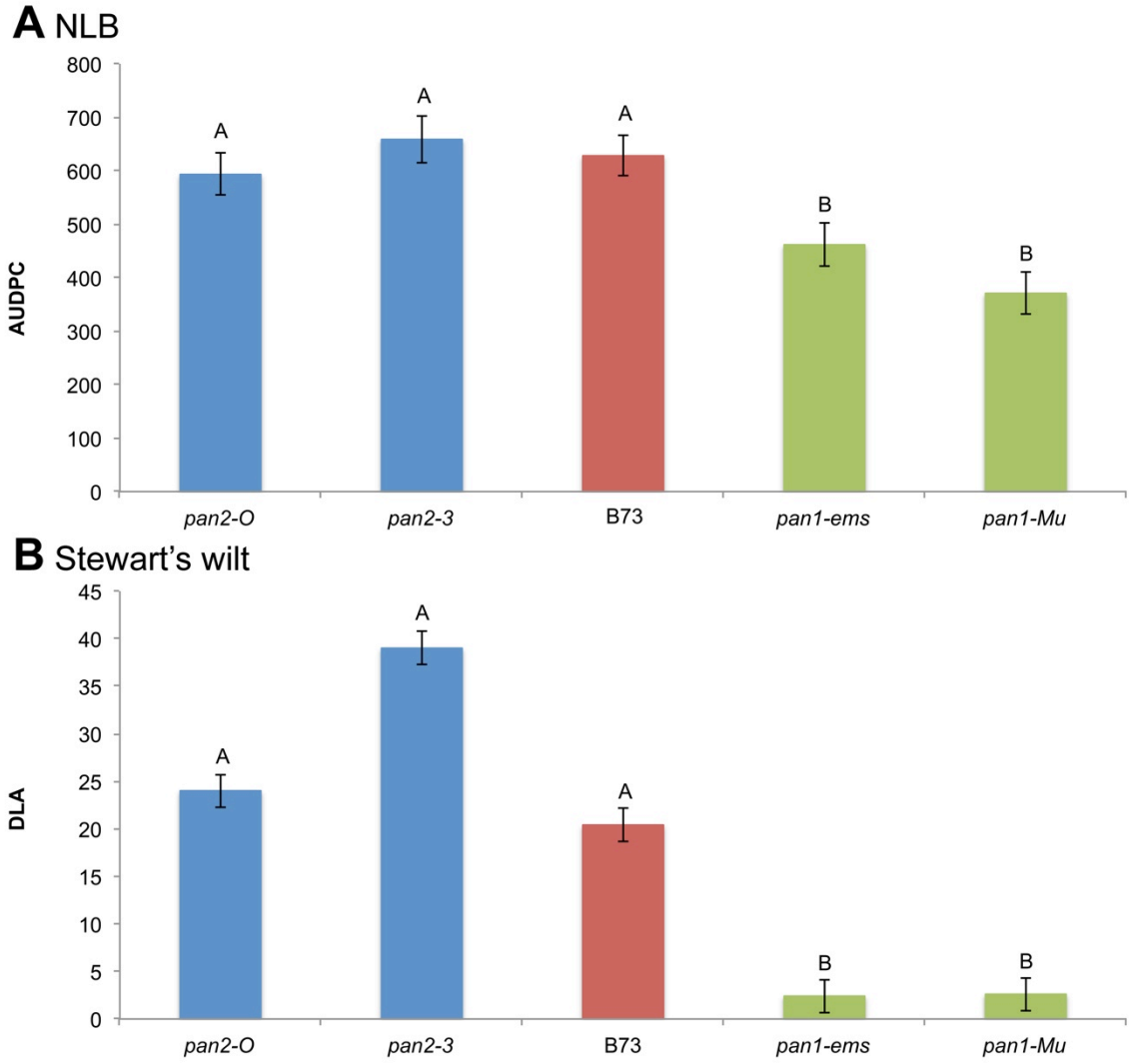

Figure 6. Disease phenotypes of *pan* mutants. Plants homozygous for both *pan1-ems* and *pan1-Mu* alleles are significantly different from B73, the background for the mutants, while *pan2* mutants are not. Letters denote significance with Student's *t*-test (*p*-value <0.0001). *pan2* mutants are shown in blue, while pan1 mutants are shown in green. B73 is shown in red.

Table 1. Markers. The markers used in this fine-mapping study, along with their physical positions (AGP_V2) and primer sequences are listed. Locus names are derived from marker available from panzea.org (begin with PZA or ss) or derived for this study (begin with chr1 or ch1). Physical positions are AGP_V2 postions.

Marker ID	Locus name	Physical position	Forward primer 1	Forward primer 2	Common reverse primer
snp_01_0042	ch1_AC202158_78820	180,394,890	GAAGGTGACCAAGTTCATGCTGCCGATACCAATCACTGACA	GAAGGTCGGAGTCAACGGATTCTGCCGATACCAATCACTGACG	GCCCCGGTCGGTACACAGTTA
snp_01_0057	chr1|183107525|C/T	184,016,638	GAAGGTGACCAAGTTCATGCTCCCTCACCAGCGACCACC	GAAGGTCGGAGTCAACGGATTCTTCCCTCACCAGCGACCACT	GGCAGGCAAGGTCACAGAGGAA
snp_01_0015	PZA00068.1	183,986,082	GAAGGTGACCAAGTTCATGCTGACAGGGAACCGGATTCTATAG	GAAGGTCGGAGTCAACGGATTGTGACAGGGAACCGGATTCTATAA	GTTAATCTTCACCTGGTGCATCGTGTA
snp_01_0059	chr1|184516928|G/A	184,633,349	GAAGGTGACCAAGTTCATGCTCCAGGGCCTGCTAACGCTGTT	GAAGGTCGGAGTCAACGGATTCAGGGCCTGCTAACGCTGTC	CGTACATGTGCCGCGCGTTTATATA
snp_01_0047	chr1|185582569|A/C	185,737,089	GAAGGTGACCAAGTTCATGCTGAATTGCTATTATTATATAACTCAACCCGT	GAAGGTCGGAGTCAACGGATTAATTGCTATTATTATATAACTCAACCCGG	TCGGCCATCTCGGCAACCTCAA
snp_01_0136	ss229502009	187,090,230	GAAGGTGACCAAGTTCATGCTGCAATAACCATTGAACCAACGAC	GAAGGTCGGAGTCAACGGATTCTGCAATAACCATTGAACCAACGAG	GTCCAGCTATAGGATAGGAAGAGCAT

snp_01_0137	ss229502244	187,245,104	GAAGGTGACCAA GTTCATGCTGCGT CATTTTCTCGTCA GGGC	GAAGGTCGGAGTCAA CGGATTCTGCGTCATT TTCTCGTCAGGGA	ACTCCATTATTCATG CTTGATGGACCTA
snp_01_0138	ss229502327	187,272,680	GAAGGTGACCAA GTTCATGCTACTG CTAGCAGCTACTG CAGG	GAAGGTCGGAGTCAA CGGATTCTACTGCTA GCAGCTACTGCAGA	CCAACCTTTACCTAA ACATGTTTGCTGTT
snp_01_0139	ss229502401	187,341,010	GAAGGTGACCAA GTTCATGCTGCGC CTCTCCTCCACGG C	GAAGGTCGGAGTCAA CGGATTGCGCCTCTCC TCCACGGT	ACGGTGCCCGGCGC GTGAA
snp_01_0115	ss229502466	187,399,046	GAAGGTGACCAA GTTCATGCTCGTA ACGGCAAGCTTCT CTGTGT	GAAGGTCGGAGTCAA CGGATTGTAACGGCA AGCTTCTCTGTGC	TCTCTGGCGTAGGGT CCTCTCT
snp_01_0116	ss229502486	187,400,044	GAAGGTGACCAA GTTCATGCTTCCG CTGCCGCTGCGG A	GAAGGTCGGAGTCAA CGGATTCCGCTGCCG CTGCGGG	GCAGTCGTGGCGGTC CGAGTA
snp_01_0117	ss229502506	187,401,161	GAAGGTGACCAA GTTCATGCTGGAC AGCGAACCCGGG GA	GAAGGTCGGAGTCAA CGGATTGGACAGCGA ACCCGGGGG	CCTTGGTCGCGTCTG GCTGCT
snp_01_0118	ss229502527	187,436,581	GAAGGTGACCAA GTTCATGCTTTGT TAAGCAAGCACA CAGAAGCG	GAAGGTCGGAGTCAA CGGATTTGTTAAGCA AGCACACAGAAGCA	CAGCTGCTCGTCGTC TGTTGTTAAT
snp_01_0079	ss196501884	187,588,467	GAAGGTGACCAA GTTCATGCTACCG	GAAGGTCGGAGTCAA CGGATTCCGGAACAT	CGGAGAGGATCACG CCGAAGTT

snp_01_0082	ss229504554	189,336,643	GAACATCGTCAAGATGGAA GAAGGTGACCAAGTTCATGCTCCTTGCGCTCTCAGGTTTTTGCA	CGTCAAGATGGAG GAAGGTCGGAGTCAACGGATTCTTGCGCTCTCAGGTTTTTGCG	TCATTCAGGTGGGCCCAGGCT
snp_01_0083	ss196429231	189,352,206	GAAGGTGACCAAGTTCATGCTCAGCCCCCTCTGTCCGTT	GAAGGTCGGAGTCAACGGATTCAGCCCCCTCTGTCCGTC	CCTTCAAGCTCGAGCTGGGACT
snp_01_0085	ss196516288	190,594,339	GAAGGTGACCAAGTTCATGCTGTTTAAGAATTGGATATCATTGATCGAC	GAAGGTCGGAGTCAACGGATTCTGTTTAAGAATTGGATATCATTGATCGAT	GGCGTTGCTGATATCGCGTTCAATT
snp_01_0061	chr1|193313487|C/T	193,478,836	GAAGGTGACCAAGTTCATGCTGGTTCGTCCATTGCCGGAATC	GAAGGTCGGAGTCAACGGATTCGGTTCGTCCATTGCCGGAATT	CGAACTCAACAGAGGAATTCTTACCTT
snp_01_0005	PZA00619.3	195,557,990	GAAGGTGACCAAGTTCATGCTGAAGCACTCAACGCCGCCAGA	GAAGGTCGGAGTCAACGGATTAGCACTCAACGCCGCCAGG	GMCATGCATATATATATGGCTGCCTCAT
snp_01_0088	ss229511302	196,306,976	GAAGGTGACCAAGTTCATGCTGATGTCCATGAATTTTCCAGTTCCAA	GAAGGTCGGAGTCAACGGATTGATGTCCATGAATTTTCCAGTTCCAT	AGCTCAGTACACTAGTAAAAATTAGGGTAA

Table 2. NLB QTL mapping studies. Previous QTL studies that identified QTL for NLB resistance at maize bin 1.06 are listed. Confidence interval (CI) locations are based on AGP_V2.

Reference	Parents	Resistance source	Population	QTL mapping	Trait	Flanking markers		Lower CI*	Upper CI*
Balint-Kurti *et al.* 2010	B73 x Mo17	B73	RIL	Composite interval mapping	AUDPC (AU06WMD)	bnlg1598	umc1396	187.8	191.1
Freymark *et al.* 1993	B52 x Mo17	B52	$F_{2:3}$	Interval mapping	Average Number of lesions/leaf	umc157	umc67	12.2	175.6
					Average percentage leaf tissue diseased	umc157	umc67	12.2	175.6
Welz *et al.* 1999	D32 x D145	D32	F_3	Composite interval mapping	% diseased leaf area	csu61b	dup12 (dupssr12)	181.0	239.6
Wisser *et al.* 2008	-	-	Recurrent selection	Selection mapping	-	bnlg615		201.0	
Chung *et al.* 2010	B73 x Tx303	Tx303	NILs	-	IP, AUDPC	umc1754	umc2234	180.0	187.4
Chung *et al*. 2011	B73 x CML52	CML52	HIFs	-	IP, lesion number, diseased leaf area, AUDPC			182.6	189.8
	B73 x CML52	CML52	RIL	ICIM	3 diseased leaf area ratings			200.4	205.8
Zwonitzer *et al.* 2010	Ki14 × B73	Ki14	RIL	Multiple interval mapping	sAUDPC	PZA010 41.2	bnlg1057	157.1	190.0

Poland *et al.* 2011	NAM	B97 CML103 CML247 CML52 CML69 Ki11 Ki3 M37W Mo17 Mo18W NC358 Tzi8	NAM	Joint linkage mapping	AUDPC	PZA02191.1	PZA00619.3	182.5	195.6

Table 3. Candidate genes. Candidate genes that fall within the NLB fine-mapping interval.

Gene ID	Start (AGPV2)	Stop (AGPV2)	Interpro Description
GRMZM2G119511	185735951	185738575	Pyridoxal phosphate-dependent transferase
GRMZM2G419430	185737836	185738414	Calcium-binding EF-hand
GRMZM2G419431	185739064	185740761	
GRMZM2G419436	185739619	185740699	Serine/threonine-protein kinase, active site
GRMZM2G119547	185741603	185742128	Actin cross-linking
GRMZM2G124428	185777384	185779013	Wall-associated receptor kinase galacturonan-binding domain
AC190935.2_FG001	186007538	186008047	
GRMZM2G116254	186214773	186215342	Calcium-binding EF-hand
GRMZM2G552586	186230672	186232165	Aldehyde/histidinol dehydrogenase
GRMZM2G116236	186236715	186237201	
GRMZM2G417360	186237596	186238072	HAT dimerisation
AC186416.3_FG001	186340690	186342603	
GRMZM2G037493	186468876	186472999	SANT/Myb domain
GRMZM2G037581	186533673	186537407	WD40 repeat
GRMZM2G502940	186538248	186538450	
AC215187.3_FG003	186593280	186593915	
AC205695.3_FG008	186615110	186616941	
GRMZM2G362303	186633675	186639255	Protein kinase, catalytic domain
GRMZM2G308597	186640159	186640742	Calcium-binding EF-hand
GRMZM2G449226	186652945	186655618	
GRMZM2G391281	186674312	186675233	
GRMZM2G391288	186675613	186678029	S-receptor-like serine/threonine-protein kinase
GRMZM5G811972	186678239	186678787	
AC208564.3_FG004	186736360	186737646	Transcription factor, K-box
GRMZM2G061739	186964841	186965674	
AC211887.3_FG001	186964865	186965437	
GRMZM5G832154	186972934	186973635	
GRMZM5G800323	186973040	186973858	
GRMZM2G061791	186995763	186999056	
GRMZM2G359559	187002125	187004115	Aminotransferase, class V/Cysteine desulfurase
AC211887.3_FG004	187003702	187004115	Calcium-binding EF-hand

GRMZM2G059012	187055101	187062082	Wall-associated receptor kinase galacturonan-binding domain
AC211887.3_FG006	187063931	187064597	
GRMZM2G359434	187065602	187069942	Pentatricopeptide repeat
AC211887.3_FG007	187065698	187066120	Calcium-binding EF-hand
GRMZM2G059129	187091607	187095345	Glycerophosphoryl diester phosphodiesterase
GRMZM2G085210	187118709	187121135	Proton-dependent oligopeptide transporter family
GRMZM2G119381	187179081	187180794	
GRMZM2G419267	187190806	187194254	Glycosyltransferase AER61, uncharacterised
GRMZM2G445684	187269130	187271929	Zinc finger, C2H2
GRMZM2G445676	187271948	187272122	
AC213857.4_FG001	187338417	187341110	
GRMZM2G083755	187341766	187344871	Frataxin/CyaY
GRMZM2G083803	187344872	187345544	
AC213857.4_FG003	187344907	187345239	EF-Hand 1, calcium-binding site
GRMZM2G552850	187353437	187354156	
AC212463.3_FG001	187368059	187379099	
GRMZM5G834455	187379121	187379438	
GRMZM2G142507	187380518	187384704	
GRMZM2G441888	187383447	187384622	Photosystem II PsbP, oxygen evolving complex
GRMZM2G142597	187387273	187397432	RNA recognition motif domain
GRMZM2G441903	187398921	187400738	Zinc finger, AN1-type
GRMZM2G142638	187403377	187407974	Poly(A) polymerase, central domain
AC212463.3_FG009	187435427	187436221	
GRMZM2G020478	187441277	187443050	Serine/threonine- / dual specificity protein kinase, catalytic domain
GRMZM2G132763	187587612	187591058	Leucine-rich repeat
GRMZM2G132748	187626539	187627195	NADH:ubiquinone oxidoreductase, ESSS subunit
GRMZM2G132704	187635249	187640520	Dilute
GRMZM2G132623	187641040	187642585	Ribosomal protein L31e
GRMZM2G435224	187641060	187642612	
GRMZM2G132607	187643004	187646940	Carbohydrate kinase PfkB
GRMZM2G563405	187649672	187650116	
GRMZM2G141320	187667515	187670309	Diacylglycerol glucosyltransferase, N-terminal
GRMZM2G040129	187753619	187755711	DNA-directed DNA polymerase, family B, mitochondria/virus

GRMZM2G580853	187842642	187843102	
GRMZM2G163771	187843888	187844570	
GRMZM2G163783	187845063	187845576	
GRMZM2G121302	187877315	187877869	Cyclophilin-like peptidyl-prolyl cis-trans isomerase domain
GRMZM5G873791	187877340	187877853	
GRMZM2G121312	187883668	187885516	Leucine-rich repeat-containing N-terminal, type 2
GRMZM2G121398	187889142	187892150	
GRMZM5G839014	187975033	187977356	Ovarian tumour, otubain
GRMZM5G836190 (*pan1*)	187978007	187980515	Leucine-rich repeat-containing N-terminal, type 2
GRMZM5G854901	188014689	188017810	tRNA/rRNA methyltransferase, SpoU
GRMZM5G879527	188031610	188034600	Myc-type, basic helix-loop-helix (bHLH) domain
GRMZM2G703846	188036995	188037538	
GRMZM5G861100	188059257	188062957	
AC234203.1_FG009	188060402	188062129	rRNA-processing protein EFG1
AC234203.1_FG010	188063594	188066385	
AC234203.1_FG011	188071750	188073648	Ethylene insensitive 3-like protein, DNA-binding domain
AC234203.1_FG004	188083185	188089513	ABC transporter, transmembrane domain
AC234203.1_FG005	188092362	188092850	
GRMZM5G822593	188114875	188119970	Lipoxygenase, LH2
GRMZM2G161004	188169374	188172490	G-patch domain
GRMZM2G160917	188181863	188185970	Transcription factor, SBP-box
GRMZM2G159263	188256572	188256759	
GRMZM2G413230	188333139	188333371	
GRMZM2G115436	188342737	188344114	
GRMZM2G115442	188344346	188355656	Short-chain dehydrogenase/reductase SDR
GRMZM2G115462	188386434	188386882	Ribonuclease T2-like
GRMZM2G022499	188457694	188461344	SANT/Myb domain
GRMZM2G403669	188600179	188600736	
GRMZM2G403667	188601086	188602301	
GRMZM2G403664	188601935	188602807	
GRMZM2G007681	188719663	188811722	RNA polymerase, N-terminal
GRMZM2G325543	188733228	188736419	Zinc finger, BED-type predicted
GRMZM2G430455	188855847	188859402	KOW
GRMZM2G130659	188880026	188886014	Nonaspanin (TM9SF)
GRMZM2G430522	188906106	188908967	No apical meristem (NAM)

			protein
GRMZM2G467263	188927396	188928192	
GRMZM2G168669	188928450	188929193	
GRMZM2G342437	188983389	188985663	
GRMZM2G042622	188986180	188988366	Serine-threonine/tyrosine-protein kinase catalytic domain
GRMZM5G847243	189045340	189045985	
GRMZM2G357919	189077793	189078897	Protein kinase, catalytic domain
GRMZM2G055992	189081768	189085843	Leucine-rich repeat
GRMZM2G056056	189088108	189090646	
GRMZM2G056122	189091152	189093163	Pentatricopeptide repeat
GRMZM2G088627	189145769	189150346	Peptidase M20
GRMZM2G009009	189188469	189190473	
GRMZM2G009117	189191520	189193031	Heavy metal-associated domain, HMA
AC186691.4_FG009	189196177	189197677	
GRMZM2G009154	189200093	189201905	Glycosyl-phosphatidyl inositol-anchored, plant
GRMZM2G308873	189200780	189201223	
GRMZM2G158182	189276877	189277368	
GRMZM2G458441	189278497	189283529	Peptidase C54
AC186691.4_FG003	189334539	189334808	

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CHAPTER 3

MULTIPLE GENES, INCLUDING A PUTATIVE REMORIN, ARE IMPLICATED IN QUANTITATIVE DISEASE RESISTANCE AGAINST DIVERSE PATHOGENS OF MAIZE[3]

Abstract

The mechanisms of quantitative disease resistance remain largely elusive and the basis of multiple disease resistance undetermined. In this study, we chose to fine-map a known quantitative disease resistance locus in maize, *qNLB1.02,* which confers resistance to three foliar diseases of maize: northern leaf blight (NLB), caused by *Setosphaeria turcica*; Stewart's wilt, caused by *Pantoea stewartii*; and common rust, caused by *Puccinia sorghi*. *qNLB1.02* provides broad-spectrum resistance to *S. turcica*, and *qNLB1.02* has been previously shown to provide protection against vascular invasion by this pathogen. Using a population derived from previously characterized introgression lines, a 27-Mb interval was found to have multiple QTL for NLB resistance. The common rust QTL co-localized with the Stewart's wilt QTL in a 5.26-Mb interval, while the 2.56-Mb interval for rust resistance overlapped with the Stewart's wilt QTL. Positional candidate genes from the fine-mapping interval were identified and investigated. The gene *roughsheath2-interacting KH domain protein* (*rik*) (Phelps-Durr *et al*., 2005; Buckner *et al.,* 2008) was tested as a putative candidate for *qNLB1.02* based on initial fine-mapping and association mapping. *rik* was found to be up-regulated during infection by *S. turcica,* but was not significant in

[3] Jamann, T, Luo, X, Morales, L, Kolkman, J., Chung, C, Nelson, R. Multiple genes, including a putative remorin, are implicated in quantitative disease resistance against diverse pathogens of maize. In preparation.

a subsequent association analysis and was excluded based on subsequent fine-mapping. Four candidate genes selected based on physical position within the narrowed fine-mapping interval were examined. Two genes, a putative remorin and a putative F-box gene, were found to be more highly expressed in the resistant line. Mutants were tested for two of the four genes and the putative remorin showed an effect, strongly suggesting this putative remorin underlies the QTL.

Introduction

Quantitative disease resistance (QDR) has frequently been employed through conventional plant breeding, and many studies examining the genetic architecture of quantitative resistance identified only a few loci contributing to resistance (Van der Plank, 1984). Using a large, multi-parental maize population, a highly complex genetic architecture for QDR has been revealed (Kump *et al.,* 2011; Poland *et al.,* 2011). For southern leaf blight (SLB) and northern leaf blight (NLB), 32 and 29 independent loci, respectively, have been implicated in quantitative resistance (Kump *et al.,* 2011; Poland *et al.,* 2011). Through genome-wide association studies with multiparental populations and diversity panels a sizeable number of candidate genes have emerged (Chia *et al.,* 2012; Schaefer and Bernardo, 2013; Van Inghelandt *et al.,* 2012). However, these genes have not been validated and the mechanisms underlying QDR remain largely a matter of speculation (Poland *et al.*, 2009).

A number of mechanisms have been postulated to be involved in QDR, including genes involved in avoidance, perception, and signaling (Poland *et al.,* 2009). Although many genomic regions have been associated with incomplete resistance,

these regions are typically associated with hundreds of genes. The few genes that have been demonstrated to play a role in QDR illustrate the diversity of mechanisms that influence the trait. The known QDR genes include an ABC transporter, a kinase-START gene, a cluster of germin-like proteins, and a proline-rich protein (Fu *et al.,* 2009; Fukuoka *et al.,* 2009; Krattinger *et al.,* 2009; Manosalva *et al.,* 2009). These genes implicate a broad range of mechanisms (St Clair, 2010). Further cloning of loci will elucidate the mechanisms that underlie QDR and provide additional insight into their diversity.

QDR can vary in specificity, from providing protection against one race of a pathogen, to providing protection against diverse microbes (St Clair, 2010). Many regions of the genome have been associated with resistance to diverse diseases, but the low resolution of these mapping studies has not allowed linkage to be distinguished from pleiotropy (Wisser *et al.,* 2006). Only in a few instances has a gene been demonstrated to confer pleiotropic resistance. Proteins at highly connected nodes of the proteome are targets of effectors from diverse taxa, as are those related to plant hormones (Mukhtar *et al.,* 2011). The mechanism of resistance may provide some insight into the specificity of resistance conditioned by a gene. For example, regulatory genes, such as those related to hormones, have been shown to provide protection against diverse pathogens (Todesco *et al.,* 2010). In other cases, the mechanisms of pleiotropic resistance remain obscure, as in the case of the putative ABC transporter encoded by *Lr34*, which confers durable resistance to two rusts and powdery mildew (Krattinger *et al.,* 2009). One QTL in maize that has been consistently mapped as effective against diverse diseases, such as northern leaf blight

(NLB), southern leaf blight, common rust, Stewart's wilt, and ear and stalk rots by multiple fungal pathogens, has been mapped to bin 1.02 (referred to as $qNLB1.02_{B73}$) (Chung *et al.,* 2010b; Wisser *et al.,* 2006).

NLB is a foliar disease of maize caused by the *Setosphaeria turcica* (anamorph=*Exserohilum turcicum*) (Luttrell) K.J. Leonard and E.G. Suggs. In inoculated field trials, yield losses of up to 63% have been associated with severe NLB epidemics (Perkins, 1987; Pingali and Pandey, 2001; Raymundo, 1981). Disease progression is favored by moderate temperatures and high humidity, and NLB is thus endemic in many regions of the world (Adipala *et al.,* 1993; Fininsa and Yuen, 2001; Levy and Pataky, 1992). Stewart's wilt, a seed-borne disease caused by *Pantoea stewartii*, can be highly prevalent in years when corn flea beetle pressure is high and is important in part because of zero-tolerance phytosanitary requirements (Esker and Nutter, 2002; Khan *et al.,* 1996). Both pathogens are hemibiotrophs and once inside the plant, they spread through the vascular tissue, causing wilted lesions by plugging xylem vessels (Jennings and Ullstrup, 1957; Roper, 2011). The third disease for which this locus is associated with resistance against is common rust, caused by *Puccinia sorghi*. Common rust is especially important in sweet corn production, where yields can be decreased up to 49% by rust (Groth *et al.,* 1983).

Maize bin 1.02 has been identified as a pleiotropic locus in maize with effects on traits such as disease resistance and flowering time. Nested association mapping for NLB identified a QTL at 1.02 with joint linkage mapping. A positive relationship between flowering time and disease severity has been noted in maize, where lines that flower later are more resistant (Wisser *et al.,* 2011). When examined at the QTL level,

this correlation tends to dissipate (Poland *et al.,* 2011). The QTL at 1.02 was the only QTL to show a correlation with flowering time, where there was an inverse correlation with flowering time, indicating a potential role for pleiotropy at the locus (Poland *et al.,* 2011). In order to dissect the resistance conditioned by this locus, Chung *et al.* (2010b) used introgression lines to infer the mechanism of resistance conditioned by the locus and found that the QTL acted to slow *S. turcica* pathogenesis between the initial penetration phase and vascular invasion. Introgression lines contrasting for NLB also contrasted for common rust and Stewart's wilt, and B73 was the resistance donor for all three diseases (Chung *et al.,* 2010b).

In order to understand the genetic relationship(s) underlying broad-spectrum resistance at a single locus and to identify genes conditioning resistance, we used high-resolution fine-mapping, complemented by association mapping, expression analysis and mutant analysis. We were able to separate resistance to rust and Stewart's wilt from resistance to NLB, but were unable to separate resistances for rust and Stewart's wilt. We demonstrate a role for a putative F-box encoding gene in NLB resistance, and provide strong evidence that a putative remorin is one of multiple genes underlying this quantitative disease resistance QTL.

Materials and methods

Plant materials

The QTL at 1.02 for NLB, Stewart's wilt and common rust was identified and confirmed previously (Chung *et al.,* 2010b). The fine mapping population was derived from the TBBC3 (**T**x303 x **B**73 **B**ack**c**ross **3**) population, a population of

chromosomal segment introgression lines composed of Tx303 introgressions in a B73 background (Szalma *et al.,* 2007). TBBC family 42_10E (Chung *et al.,* 2010b) was crossed with B73 and seed from 22 heterozygote F_2 individuals was planted at Cornell's Robert Musgrave Research Farm in Aurora, NY in 2009. A total of 3,328 plants were screened for recombinants in 2009 and an additional 1,631 recombinants in 2011. Recombinants (n=1,239) were identified between snp_01_0031 and snp_01_0026, representing the flanking markers for *q1.02* in 2009 and recombinants (n=230) were identified between snp_01_0067 and snp_01_0028 in 2011. Homozygous recombinants were evaluated for NLB, Stewart's wilt and common rust at Aurora, NY in 2010, 2011, 2012, and 2013, respectively.

Disease evaluations

Northern leaf blight

A single race 1 isolate, StNY001 (Chung *et al.,* 2010a), collected in Freeville NY in 1983, was used for all disease trials. For race testing, races 0, 1, 23, and 23N (Leonard *et al.,* 1989) were used, represented by isolates St10a, StNY001, St86A, and St28A, respectively (Chung *et al.,* 2010a). Race testing was conducted in the greenhouse at Cornell University in Ithaca, NY. Field NLB disease trials were carried out at Cornell's Robert Musgrave Research Farm in Aurora, NY from 2009-2013. An incomplete block design was used for all fine-mapping trials. A complete block design was used for mutant analysis. Inoculations were conducted as previously described (Chung *et al.,* 2010a). Briefly, both a spore suspension and solid inoculum were used to ensure infection in all weather conditions in the field, while only spore suspension

was used for greenhouse trials. For the spore suspension, *S. turcica* isolates were cultured on lactose-casein agar for two to three weeks at 25°C with a 12 hour/12 hour light/dark cycle. A spore suspension was prepared by flooding plates with 5 mL ddH_20 and using a glass rod to dislodge conidia. The resulting suspension was filtered through two layers of sterilized cheesecloth. Conidial concentration was adjusted to $4x10^3$ per mL using a haemocytometer, and the suspension was brought to a final concentration of 0.02% Tween 20. Spore suspension (0.5 mL) was introduced into the whorl of 5-6 leaf stage plants for both field and greenhouse inoculations. Field inoculations were supplemented with solid inoculum consisting of autoclaved sorghum grains cultured with *S. turcica* for 2-3 weeks at 25°C with a 12 hour light/dark cycle (Chung *et al.,* 2010b).

Plants in the field were scored for diseased leaf area (DLA) about 3-4 days after flowering and then two subsequent times, approximately 7 days after the previous scoring. Disease was scored on a row basis using a scale from 0-100 with single integer units, with 0 indicating no disease and 100 indicating complete disease. Using these three ratings, the area under the disease progress curve (AUDPC) was calculated (Chung *et al.,* 2010b; Wilcoxson *et al.,* 1974). For greenhouse trials, the percentage of leaf area that was necrotic from primary infections (PrimDLA) was scored with 1% increments.

Stewart's wilt

Stewart's wilt trials were carried out at Cornell's Robert Musgrave Research Farm in Aurora, NY in 2010, 2011, and 2012. Inoculations were carried out as described by

Chung *et al.* (2010a). Briefly, nutrient broth was inoculated with a stock culture of *Pantoea stewartii* isolate PsNY003. Inoculum was prepared as previously described (Chung *et al.*, 2010a). Prick inoculations were conducted on five- to six-leaf stage plants (Chung *et al.*, 2010a). Disease was scored on a row basis at two to three weeks post inoculation using a scale from 0-100 with single integer intervals, with 0 indicating a plant without any disease, and 100 indicating a completely diseased plant.

Common rust

Urediniospores of *Puccinia sorghi* were collected in Aurora, NY in 2007. Rust field trials were carried out at Cornell's Robert Musgrave Research Farm in Aurora, NY in 2010, 2011, and 2012. Rust inoculations were carried out as previously described (Chung *et al.,* 2010a). Briefly, about one month before field inoculations, spores were increased by inoculating susceptible sweet corn seedlings at the 3-4 leaf stage in the greenhouse. About 200-300 mg of urediniospores were suspended in 100 mL of Sortrol oil (Chevron Phillips Chemical Company, Borger, TX, USA) and sprayed onto leaves using a spray gun. Plants were kept at >80% humidity overnight. Urediniospores were collected about 3 weeks later by agitating infected sweet corn leaves in water and filtering the spore suspension through four layers of cheesecloth. Field trials were inoculated with 1.0 mL of spore suspension ($2.0x10^5$ urediniospores in 0.02% Tween 20) in the whorl. Disease was scored on a per row basis three times, beginning 3 weeks after inoculation with ratings every 10 days. A scale of 0-9 with 0.5 increments was used, where 0 indicated no disease and 9 indicated complete disease. The AUDPC was calculated, as previously described (Chung *et al.*, 2010b).

Genotyping assays

DNA extraction

High-throughput DNA extractions were used to identify recombinants, and high-quality CTAB DNA extractions were performed for all other applications. The high-throughput extraction was performed using ExNAmp (Sigma-Aldrich, St. Louis, MO, USA). Leaf tissue (1 mm^2) was plated into 0.2-mL PCR tubes, and 8 µl of extraction buffer was added to each sample. Samples were then incubated at 95°C for 10 minutes, and 8 µl of dilution buffer was added to the samples. The DNA was diluted 1:100 for KASPar genotyping assays.

The high-quality CTAB extractions were performed as previously described (Chung *et al.,* 2010a; Doyle and Dickson, 1987). Briefly, 0.1 mg of plant tissue was loaded in 96 well plates (Corning® Costar 96 Well Polypropylene Cluster Tubes) with a stainless steel grinding ball (OPS Diagnostics, Lebanon, NJ, USA) and frozen at -80°C. Samples were pulverized using a Genogrinder 2000 and suspended in 500 µl of CTAB buffer [2% (w/v) hexadecyltrimethlyammoniµM bromide, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), and 0.2% (v/v) of 2-mercaptoethanol added prior to use]. Plates were incubated at 60°C for 15 minutes, followed by an addition of 400-µl 24:1 chloroform:isoamyl alcohol. Plates were inverted 50-70 times and centrifuged at 5,200 rpm for 15 minutes. The aqueous layer was transferred to new tubes, and 300-µl isopropanol was added. Samples were stored at -20°C overnight. Samples were spun at 5,200 rpm for 12 minutes, washed with 300-µl 70% ethanol, centrifuged for 5 minutes at 5,200 rpm, decanted, and washed with 300-µl

100% ethanol. DNA pellets were suspended in 100-120 µl of TE buffer (10-mM Tris-HCl, 1-mM EDTA, pH8.0).

Marker development

Single-nucleotide polymorphism (SNP) identification

Three approaches were used to identify polymorphic markers between the inbred lines 'B73' and 'Tx303'. Available SNPs from the Maize Diversity Project were queried using the 'Find Polymorphisms Between Two Inbred Lines' search available at http://www.panzea.org (2009; Canaran *et al.,* 2008). SNPs polymorphic between B73 and Tx303 were selected from the following resources: the original NAM genetic map marker set (McMullen *et al.,* 2009), the first-generation haplotype map of maize (Gore *et al.,* 2009), and the second-generation haplotype map of maize (Chia *et al.,* 2012). Further polymorphisms between B73 and TBBC_42 were identified using an Illumina MaizeSNP50 Beadchip (Illumina, San Diego, CA, USA) run at the David H. Murdock Research Institute, Kannapolis, NC, USA. DNA from TBBC 42_10E_02 and B73 was sent and an Excel file with names, positions, and nucleotide information for approximately 50,000 maize loci was returned for B73 and TBBC_42. The spreadsheet was queried for polymorphisms between B73 and TBBC_42, and the position of the Tx303 introgression was estimated from these data.

SNP genotyping

Competitive PCR was utilized to perform all SNP genotyping using KASPar (LGC Genomics, Beverly, MA, USA) as described by Jamann *et al.* (2014). All markers

used for this study can be found in Table 1.

QTL mapping

For NLB, best linear unbiased prediction (BLUPs) were calculated for each year by fitting a mixed model using the with AUDPC as an independent variable, and replication, block nested within replication and line as random factors using R (R Core Development Team, 2013). For both Stewart's wilt and common rust, data for all three years were analyzed together using AUDPC or DLA as an independent variable, year, and replication nested within year, block nested within replication and year, and line as random factors using R (R Core Development Team, 2013). Since only one rating was completed for Stewart's wilt, single ratings were substituted for AUDPC in the model. Missing genotypes were imputed based on the nearest flanking marker genotype. Lines with fewer than 15 markers and markers with fewer than 150 lines genotyped after imputation were removed from the analysis. BLUPs were then used for single marker regression using the qtl package in R (Broman *et al.,* 2003). Analysis was completed on a per-year basis for NLB and a combined basis for Stewart's wilt and common rust. 95% Bayes confidence intervals were calculated using R/qtl (Broman *et al.,* 2003).

Field trials

Field trials were conducted at Cornell's Robert Musgrave Farm in Aurora, NY. An incomplete block design was used for all fine-mapping trials. For NLB two, three, and three replications were used in 2010, 2011, and 2012, respectively. Three replications

were used for common rust and two for Stewart's wilt. A complete block design was used for mutant trials with three replications. All greenhouse trials were conducted at Cornell University in Ithaca, NY. A complete block design was used for all greenhouse trials.

Statistical analysis

Statistical analyses for race testing and mutant analysis were conducted in JMP 9.0. A mixed model was constructed with phenotype (either AUDPC for field experiments or DLA for greenhouse experiments) as the response, and 'replication' as a random factor, and genotype as a fixed factor. A Student's *t* test was performed to test for significant differences among lines.

Identification of candidate genes

Three criteria were used to develop a list of candidate genes after each season of fine-mapping results. AGP_ V2 (Schnable *et al.,* 2009) was consulted to find all annotated genes contained in the fine-mapping interval spanning from snp_01_0068 (24,225,835 bp AGPv2) to snp_01_0070 (31,860,782 bp AGPv2) after the 2010 field season. This list was later refined to include the genes in the interval spanning from snp_01_0142 (25,377,803 bp AGPv2) to snp_01_0145 (25,399,986 bp AGPv2) after the 2012 field season. All annotated genes from the working gene set were considered candidates. A further refined candidate gene list was developed after each season using the results of genome wide association mapping using the Goodman 282-line diversity panel (Wisser *et al.,* 2011) as explained below.

Association analysis

NLB disease means for the Goodman diversity panel (Flint-Garcia *et al.,* 2005), including population structure, were used for association analysis (Wisser *et al.,* 2011). Association analysis was performed using a Mixed Linear Model (MLM) (Yu *et al.,* 2006) with kinship (K matrix) and genotypic information from Olukolu *et al.,* (2013). All analyses were completed using TASSEL v4.0 (Bradbury *et al.,* 2007).

Sequence analysis of rik

Total plant genomic DNA was isolated with a CTAB extraction procedure, as described above. A 16 µl reaction was preformed with 1x PCR buffer, 1 µM forward primer, 1 µM reverse primer, 1.5 mM MgCl2, dNTPs, 1-3 units TAQ, and 20-50 ng DNA. Primers were developed based on AGP_V2 using Primer 3 (Rozen and Skaletsky, 2000). *rik* was sequenced in B73 and Tx303. Table 2 provides primer sequences. Thermocycling procedures were as follows: 95°C for four minutes, 30 cycles of 95°C for one minute, 61°C for one minute, 72°C for one minute, and one cycle of 72°C for 10 minutes. PCR products were purified with Exonuclease I and Antarctic Phosphatase (New England Biolabs, Ipswich, MA, USA) and sequenced at Cornell University's Life Sciences Core Laboratories Center. Sanger sequencing was performed using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and resolved on an Applied Biosystems 3730x1 DNA Capillary Sequencer. Sequencing results were trimmed and aligned using DNASTAR's SeqMan (Madison, WI). SNPs were extracted in TASSEL 3.0 using a

threshold of 0.01 (Bradbury *et al.,* 2007). Association analyses, as outlined above, were completed in TASSEL. Linkage disequilibrium analysis was completed using the LDheatmap package (Shin *et al.,* 2006) in R (R Core Development Team, 2013).

Expression analysis

Semi-quantitative PCR

To test for expression differences for *rik*, two lines were selected, one carrying the B73 allele and one carrying the Tx303 allele, from a single family of F_2 plants resulting from the crossing of B73 with TBBC family 42_10E. Plants were grown in the greenhouse for 29 days, at which time they were inoculated with a spore suspension of *S. turcica* isolate StNY001 and placed in a mist chamber overnight. Tissue samples were collected at 0, 12, 24, 48, and 72 hours post inoculation (hpi). Tissue (80 mg/sample) was collected and placed in liquid nitrogen. Frozen tissue was pulverized and total RNA was extracted using a Qiagen Plant Easy RNA Kit (Valencia, CA, USA). Invitrogen's One Step RT-PCR Kit (Grand Island, NY, USA) was used for cDNA synthesis and PCR product amplification. Primers from Buckner *et al.,* (2008) were used to amplify *rik* and ubiquitin. Thermocycling procedures were as follows: 55°C for 30 min, 94°C for 2 min, 35 cycles of 94°C for 15 sec, 65°C for 30 sec, 68°C for 1 min, and one cycle of 68°C for 5 min. PCR products were separated on a 1.5% agarose gel and photographed.

Real-time PCR

RNA was extracted using a Qiagen Plant Easy RNA Kit (Valencia, CA, USA) and

cDNA was synthesized using Invitrogen SuperScript III First strand (Grand Island, NY, USA). TaqMan primers and probes were designed using Primer Express (Life Technologies, Grand Island, NY, USA) or selected from available validated assays from Life Technologies (Grand Island, NY, USA). Validated assays Zm04021391_m1, Zm04048055_g1, and Zm04040368_g1 were used for the chaperone, remorin, and ubiquitin control, respectively. Primers and probes for other genes tested are listed in Table 2. qRT-PCR was performed with TaqMan Gene Expression master mix using standard conditions (Life Technologies, Grand Island, NY, USA) and analyzed on a ViaA7 (Life Technologies), comparing expression of the four candidate genes to the ubiquitin.

Mutant analysis

Three UfMu lines (6505, 6509 and 7948) lines were tested for NLB phenotypes. F_4 plants were self-pollinated and F_6 plants were evaluated in Aurora in 2013 for NLB. An adapted PCR protocol from Settles *et al.* (2004) was used to confirm the presence or absence of insertions. Two markers flanking the insertion were designed with Primer3 (Untergasser *et al.,* 2012). Oligonucleotides were obtained from IDT. The primers are shown in Table 2. Each reaction contained 100 ng CTAB DNA, 20 mM Tris±HCl pH 8.4, 50 mM KCl, 2 mM MgCl2, 5% DMSO, 200 mM of each dNTP, 1 mM gene-specific primer, 100 nM TIR6 primer and 1 U of Taq polymerase. Thermocycling conditions were as follows: 94°C for 1 minute; 8-10 cycles of 94°C for 25 seconds, 62°C for 30 seconds, and 72°C for 1 minute; 27 cycles of 94°C for 25 seconds, 56°C for 30 seconds, and 72°C for 1 minute; and 72°C for 5 minutes.

Products were then run and visualized on an agarose gel.

Results

Mapping of qNLB1.02

The Tx303 introgression in TBBC family 42_10E_02 was found to span from 6.1 (SYN6315) to 33.0 Mb (PUT-163a-18162870-1232) on chromosome 1 based on the Illumina MaizeSNP50 chip. This interval, however, was too large to begin a fine mapping study, so a narrowed region was selected. Previous studies showed that there was a QTL for NLB resistance at 1.01/1.02 (Chung *et al.,* 2010b), based on the TBBC3 set of near-isogenic lines, and that there was a QTL in the nested association mapping population at PZB00718.5 (chr1: 17,666,998 bp on AGPv2) (Poland *et al.,* 2011). In both of these studies, B73 was found to be the resistance donor. Based on these previous studies and the QTL mapping, the fine mapping region was selected and markers were developed. The selected flanking markers, snp_01_0031 and snp_01_0026, delimited the 14-Mb fine mapping interval, spanning from 16-30 Mb on chromosome 1.

High-resolution mapping of qNLB1.02

A mapping population of 4,959 plants was screened for recombinants. Using snp_01_0026 and snp_01_0031, recombinants (n=1,239) were identified in 2009. A total of 244 fixed lines were evaluated in 2010. An additional 1,631 plants were screened for recombinants in 2011 using snp_01_0068 and snp_01_0028, which led to

the identification of 230 recombinants. The final screening of recombinants in 2012 included those identified from both screenings. The NLB region was narrowed to snp_01_0068 and snp_01_0070 in 2010 and in 2012 the main QTL was narrowed to an interval delimited by snp_01_0142 and snp_01_0145 (Figure 1). Two distinct QTL were mapped for NLB in 2012, confirming that there are two QTL in the region, as indicated by ICIM (Poland *et al.,* 2011).

Broad-spectrum disease resistance conditioned by $q1.02_{B73}$

It was previously shown that *qNLB1.02*$_{B73}$ was also effective against Stewart's wilt and common rust (Chung *et al.,* 2010b). To determine the genetic relationship for multiple disease resistance at 1.02, the fine mapping population was evaluated for Stewart's wilt and common rust. Only 2010 and 2011 data were used for rust, as 2012 had insufficient disease pressure. The region comprising *qStw1.02* and *qRust1.02* was narrowed to 5.26 Mb (19,679,687-24,940,817 bp AGPv2) and 2.56 Mb (22,379,568-24,940,817 bp AGPv2), respectively, as shown in Figure 2.

To determine the specificity of the resistance conditioned by *qNLB1.02*, multiple races of *S. turcica* were tested for their interaction with *qNLB1.02*. When challenged with races 0, 1, 23, and 23N, this locus showed broad-spectrum resistance to NLB with respect to these races, as shown in Figure 3.

2010 Candidate genes underlying qNLB1.02$_{B73}$

qNLB1.02 encompassed a 7.65 Mb region after the 2010 season. To refine the list of candidate genes, genome-wide association mapping and genome-wide nested

association mapping results were examined (Poland *et al.,* 2011; Wisser *et al.,* 2011). One candidate emerged from the Goodman panel association mapping: *rough sheath2-Interacting KH protein* (Thoene-Reineke *et al.,* 2011) at 26.3 Mb. A SNP in *rik*, PZB01957.3, was already shown to be significant across the diversity panel (*p*-value 6.34E-05) (Kolkman, personal communication) and fell within the narrowed fine mapping region. Based upon the fine-mapping evidence and association mapping, *rik* was considered a strong candidate and investigated further.

rik: a refuted candidate and a case of an evolving analysis

rik was considered as a candidate gene for NLB due to a significant association based on an earlier association analysis and from the fine-mapping results obtained in 2010. The gene was re-sequenced for association analysis and its expression was assayed. Semi-quantitative reverse-transcription PCR showed that *rik* was up-regulated within the first 12 hours of pathogen introduction and continually up-regulated through 72 hours post-inoculation. Both the B73 and the Tx303 allele showed up-regulation in response to infection by *S. turcica,* as shown in Figure 4B. We re-sequenced *rik* in B73 and Tx303, because the original significant diversity panel SNP was not polymorphic between B73 and Tx303. We assayed the polymorphisms across the diversity panel. On average, LD broke down within 1 kb in the sequenced part of the gene, but longer range LD could be found within the gene. However, using an updated kinship matrix (Olukolu *et al.,* 2013), none of the polymorphisms in *rik* were significant, as shown in Figure 4A. While subsequent fine-mapping results ruled *rik* out as a candidate (Figure 1), the gene may still be involved in the disease response, as

indicated by the expression results.

Fine-mapping 2012

We focused on the main QTL at 25 Mb in 2012 to find underlying candidate genes. We delimited the region in 2012 to snp_01_0129 to snp_01_0028 and calculated the confidence interval of the QTL to span from snp_01_0142 to snp_01_145. Six annotated genes were identified in this 22-kb interval, including a putative pentricopeptide (GRMZM2G107805), a putative chaperonin (GRMZM2G069765), and three uncharacterized genes (GRMZM2G070442, GRMZM2G107727, and GRMZM2G523621), and a putative remorin (GRMZM2G107774). Two genes, an uncharacterized gene and a putative pentricopeptide were partially within the interval. A small gap of less than 1 kb in the reference sequence in the 22 kb segment was sequenced using the primers listed in Table 2 in Tx303 and B73. Two of the uncharacterized genes spanned the gap in the reference. Based on EST data and comparative genomics, these two uncharacterized genes spanning the reference gap shared homology with *Setaria italica* gene SI034979M.G, which has an F-box domain.

Expression analysis of fine-mapping candidate genes

We tested the expression of the genes within the narrowed fine-mapping interval in 2012, as shown in Figure 5. We found that the uncharacterized gene had very low expression and transcript levels did not significantly differ between the alleles or between pathogen-inoculated and mock-inoculated samples. The chaperonin gene was

expressed but did not show differential expression. However, we found both the putative F-box and putative remorin genes were expressed more highly in the resistant NIL carrying the B73 allele in both mock and inoculated plants with, 71-fold and 7-fold increased expression, respectively. No significant difference was found in mock- and pathogen-inoculated samples.

Mutant analysis

Three UniformMu lines with insertions in the candidate genes identified based on the 2012 fine-mapping interval were selected: one with an insertion in the chaperonin, one with an insertion in the remorin, and one with an insertion downstream of the remorin. UfMu-06509, with an insertion in the putative remorin gene, was found to be significantly more susceptible to NLB compared to W22, the background line of the mutant, as shown in Figure 6. The other two lines tested did not have homozygous mutant lines to make a comparison. We were unable to characterize a UniformMu line for *rik*, because three attempts to increase seed were unsuccessful.

Discussion

Despite its utility, the genetic and mechanistic bases of quantitative and multiple disease resistances are not well understood. In this case, we examined genomic regions associated with resistance to one biotrophic and two hemibiotrophic pathogens. Genes associated with resistance against these pathogens appear to be linked, although pleiotropy cannot be excluded for the common rust and Stewart's wilt resistance QTL. Interestingly, common rust and Stewart's wilt are caused by a fungus

and a bacterium, respectively. While the confidence intervals for the fine-mapping intervals for common rust and Stewart's wilt overlapped, the most significant marker for each disease was different. We were therefore unable to distinguish linkage from pleiotropy in this case, although the QTL peak profiles suggest tight linkage. The finding that resistance to one disease separates from the other two is consistent with other findings that pleiotropy between traits is rare in maize (Wallace *et al.,* 2014). One notable exception, however, is disease resistance (Wisser *et al.,* 2011), including potentially resistance to common rust and Stewart's wilt at this locus. This would indicate that both pathogens are targeting a common plant protein or the plant is exploiting a common microbial weakness.

We identified multiple QTL within the fine-mapping region for NLB. These findings suggest that quantitative disease resistance is even more complex than previously thought. While it was known that there are many QTL dispersed across the genome, with 29 QTL being identified in the NAM for NLB, each of these QTL likely is composed of multiple sub-QTL. Others have observed this phenomenon with disease resistance in other species (den Boer *et al.,* 2013; Johnson *et al.,* 2012), as well as other traits in maize such as domestication (Studer and Doebley, 2011). Precise phenotyping is needed to distinguish effects when a QTL with a moderate effect is broken into multiple small-effect QTL.

Association mapping initially identified *rik* as a candidate gene for NLB resistance. *rik* was within the confidence interval for the 2010 NLB fine-mapping. This was a strong candidate, as RIK interacts with RS2, the maize homolog of *AS1*. *AS1* has been described as a multiple disease resistance gene (Nurmberg *et al.,* 2007).

Additionally, *rik* is up-regulated upon infection with *S. turcica*, further suggesting a role for the gene in the interaction between maize and *S. turcica*. An updated kinship matrix with more complete genotypic information and subsequent fine-mapping ruled *rik* out as a candidate gene for the fine-mapping QTL. While this gene does not underlie this QTL, it might still play a role in other populations.

According to the joint linkage mapping in the NAM, Tx303 has a rare susceptible allele, while other inbred lines carry alleles more resistant than B73, suggesting an allelic series at 1.02 (Poland *et al.,* 2011). The discovery of multiple QTL at this region suggests that lines with resistance QTL at 1.02 may have a novel allele at one of the sub-QTL or distinct QTL. The fine-mapping interval does not overlap with the joint linkage mapping QTL from the NAM (Poland *et al.*, 2011), consistent with the hypothesis of multiple QTL at the region and supporting the interpretation that Tx303 has a rare allele.

We focused upon the QTL with the highest significance from 2010 and 2012 to identify candidate genes. The position of this QTL was confirmed in 2013. Among the candidate genes, the putative remorin and F-box gene have a previously described role in plant defense (Kim and Delaney, 2002; Lefebvre *et al.,* 2010; Raffaele *et al.,* 2009; Xie *et al.,* 1998). Expression of the F-box gene and the remorin were higher in the resistant line, but no significant differences were found in response to *S. turcica*. This suggests that structural differences in these genes might underlie this QTL.

We tested UniformMu lines with insertions in or near the remorin, and the chaperonin and found that the remorin mutant was significantly more susceptible than the corresponding line with the wild-type allele. Previously, remorins have been

shown to prevent cell-to-cell movement of PVX (Raffaele *et al.,* 2009), which is consistent with the role of this QTL in preventing entrance to the vasculature (Chung *et al.,* 2010b). Remorins have been shown to physically interact with a viral movement protein and receptor-like kinases. Two other UniformMu lines, one with an insertion downstream of the remorin and one with an insertion in the chaperonin, were increased and their NLB reaction tested, but no homozygous mutant lines were identified. The remorin and F-box gene may compliment each other in function. There is evidence that remorin proteins act as scaffold proteins that can act in signal transduction (Jarsch *et al.,* 2011), in which F-box proteins play a role (Craig and Tyers 1999). The involvement of multiple genes may increase durability and contribute to the race non-specific interaction that we observed. Further evaluation of mutant and transgenic lines are needed to definitively show a role for multiple genes.

The first step in understanding QDR is identifying the genes involved. Using fine-mapping, association mapping, expression analysis and mutant analysis, we show that this QTL is likely controlled by multiple genes, and demonstrate a role for a putative remorin in disease resistance. In this case multiple disease resistance appears to be due to linkage, although there may be a role for pleiotropy for resistance to Stewart's wilt and common rust.

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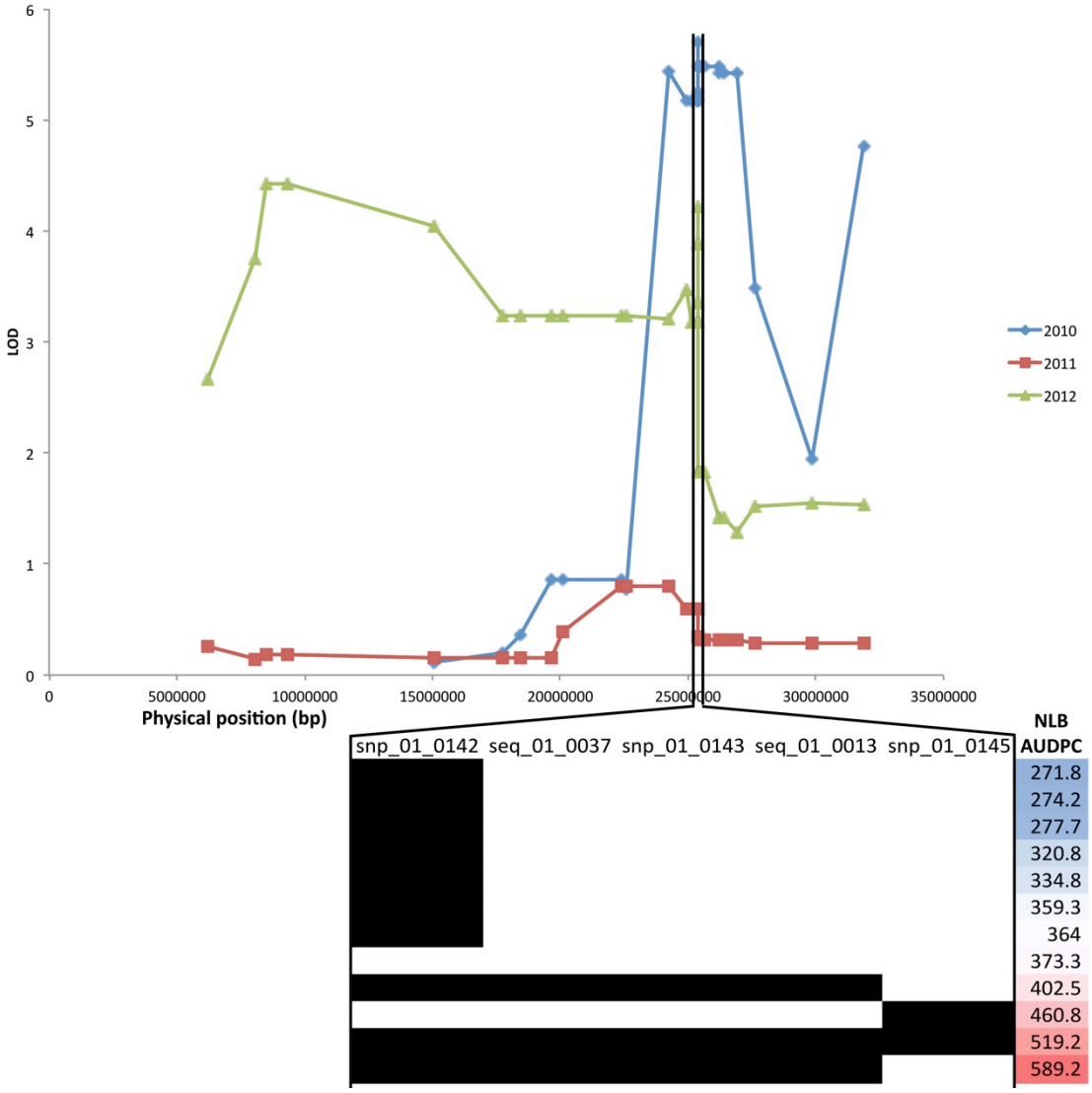

Figure 1. NLB fine-mapping breakpoint analysis. Breakpoint analysis for NLB for three years with NLB genotypes and phenotypes of recombinants tested in 2013 for narrowed region. Genotypes for the fine-mapping region from 2013 are on the left and NLB AUDPC phenotypes on the right. B73 genotypes are shown in white and Tx303 in black.

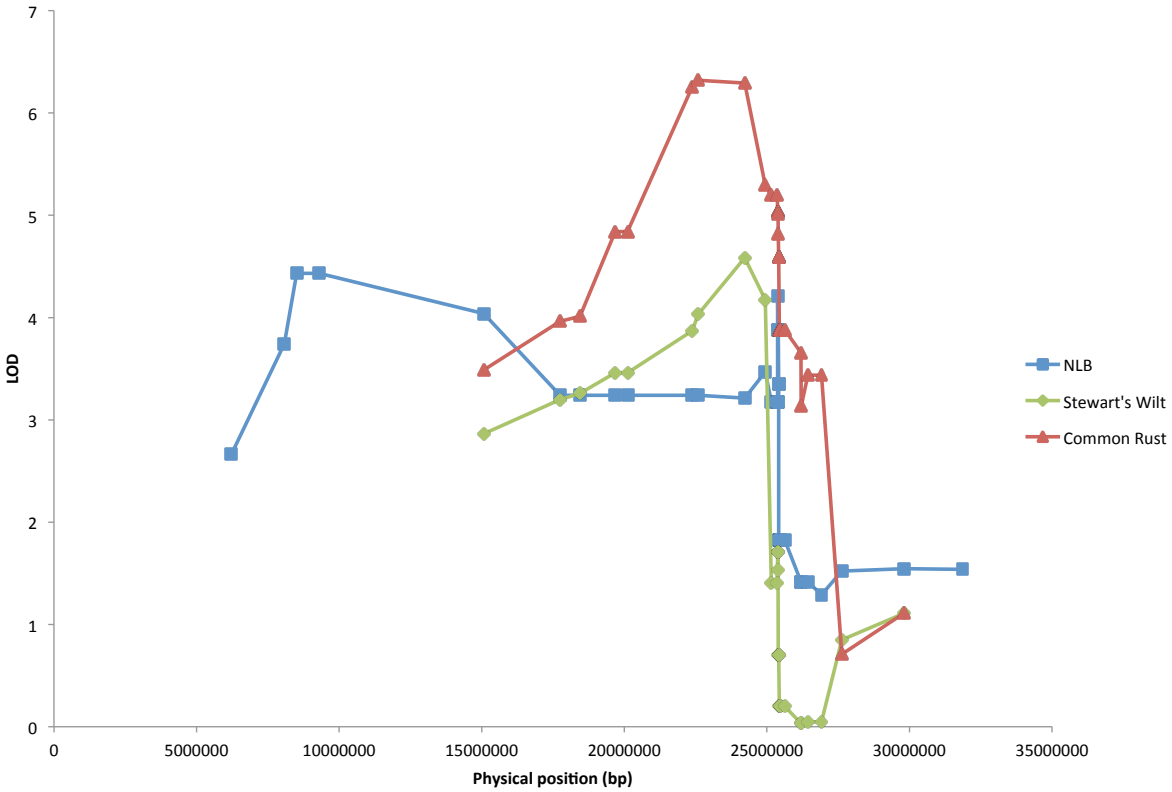

Figure 2. Multiple disease resistance fine-mapping. Breakpoint analysis for NLB (year 2012), Stewart's wilt and common rust.

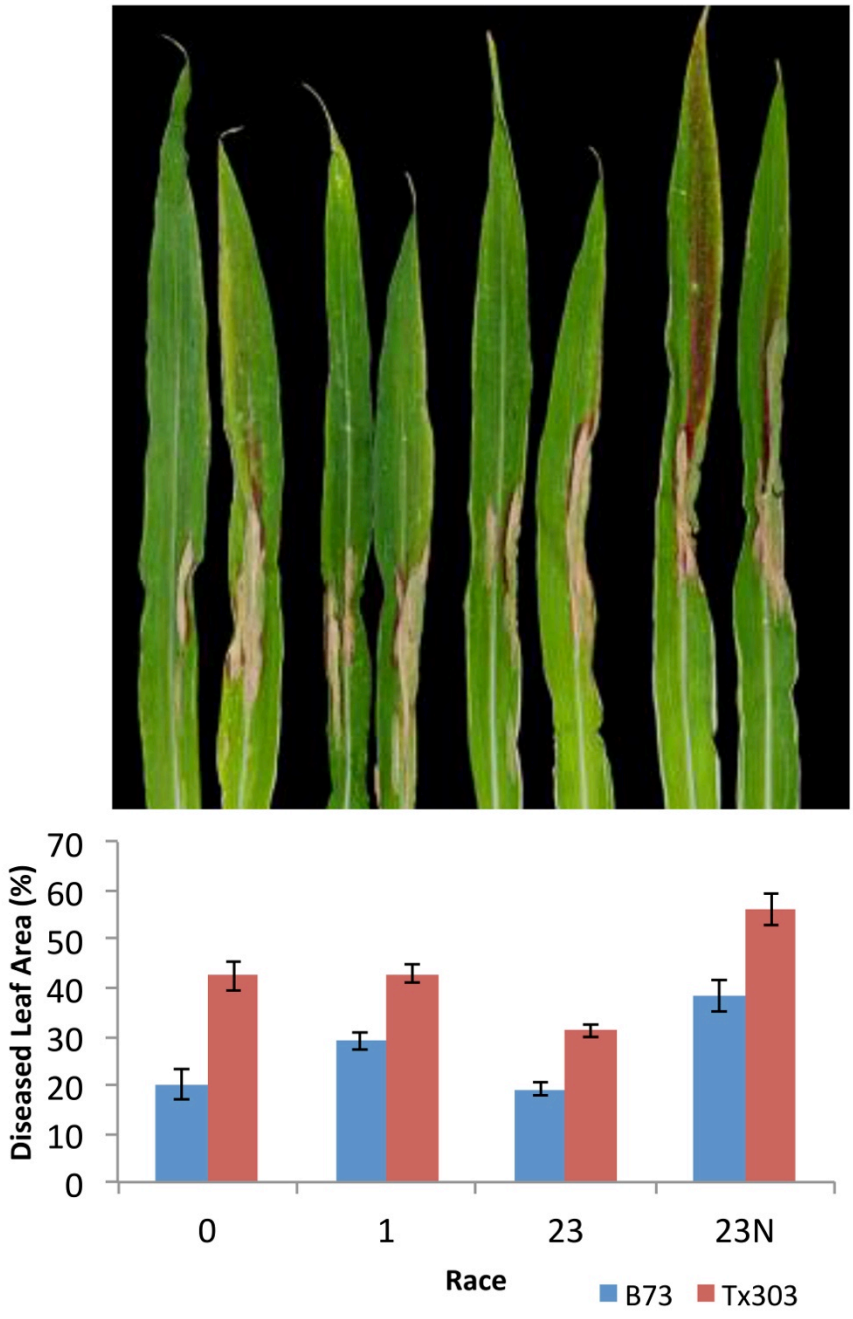

Figure 3. Race testing for *qNLB1.02*. The QTL provides broad-spectrum resistance with respect to the races tested. The B73 allele provides resistance for all races tested.

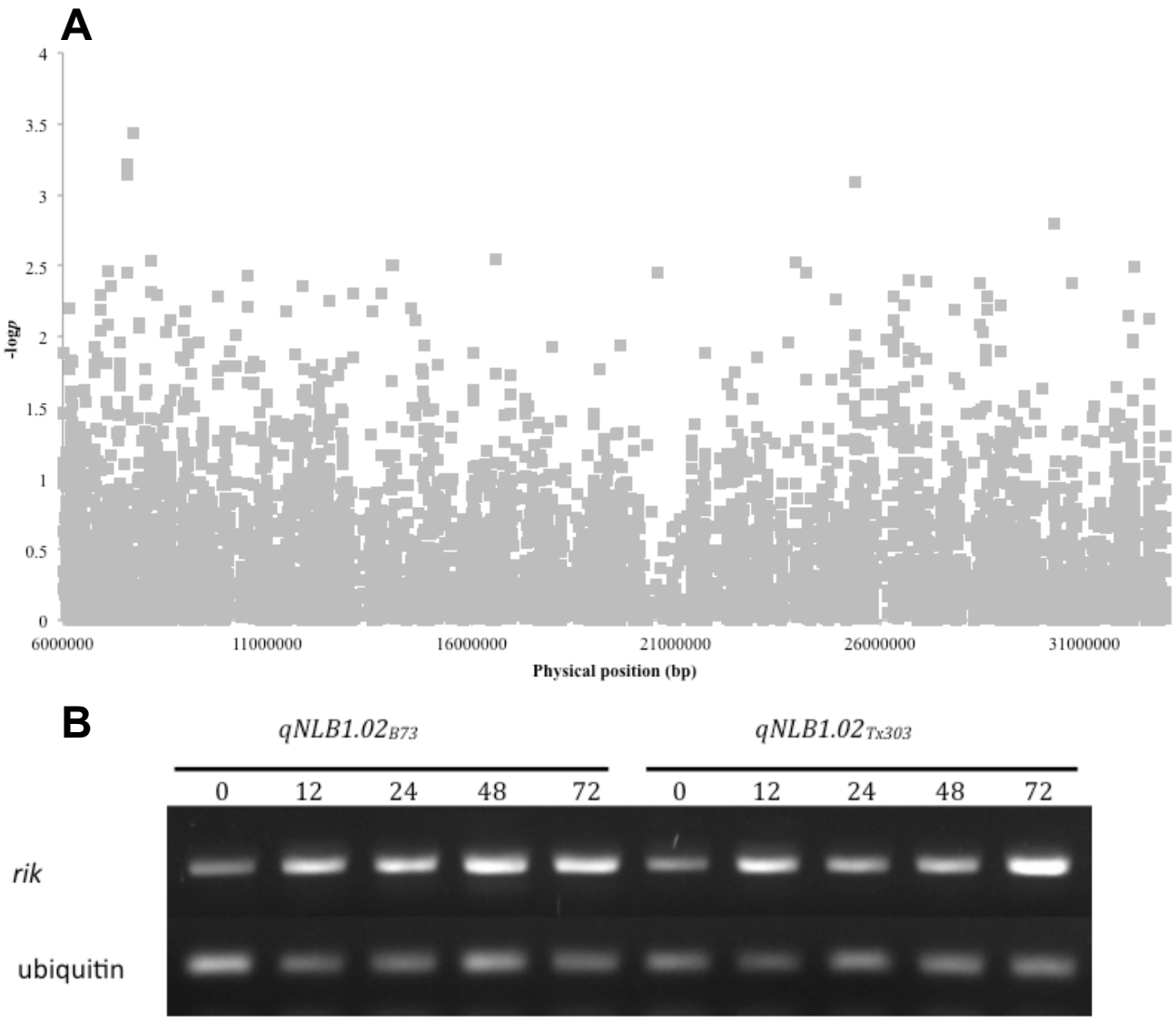

Figure 4. Association and expression analysis for *rik*. A.) Association analysis for the introgression region using the Goodman diversity panel showing no significant polymorphisms (Flint-Garcia *et al.,* 2005, Wisser *et al.,* 2011). B.) Expression analysis of *rik* showing up-regulation in both NILs after infection.

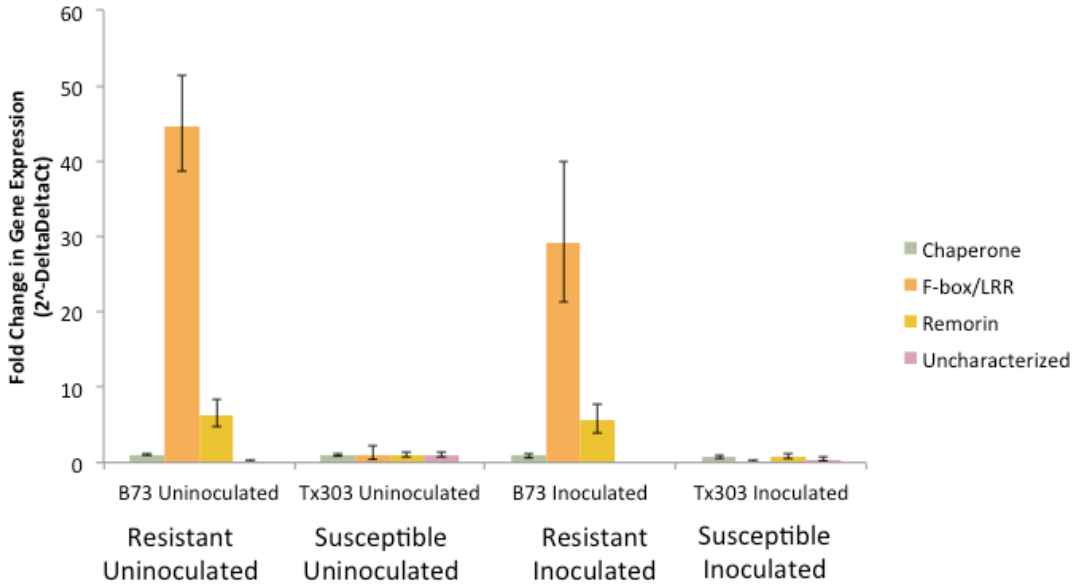

Figure 5. Expression analysis for positional candidates. Expression results for 2012 positional candidates. Expression of the putative F-box gene and the putative remorin were 71- and 6-fold higher, respectively, in the resistant NIL with a B73 allele at 1.02. No significant differences were found between uninoculated and inoculated samples.

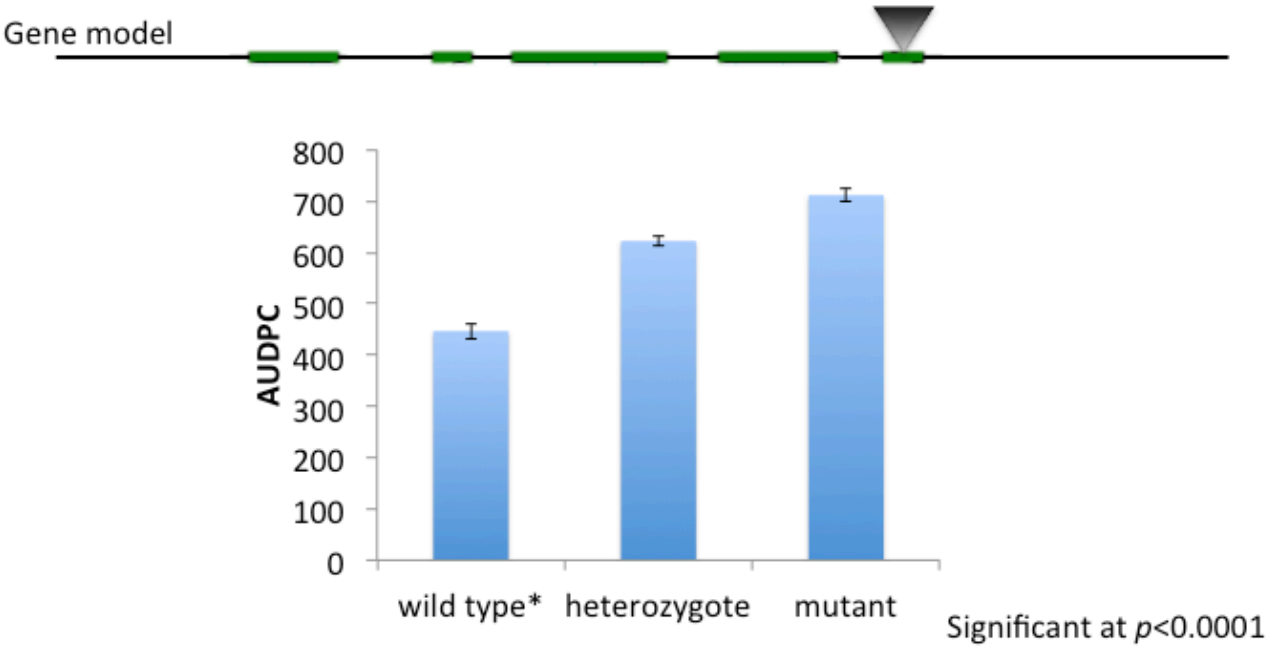

Figure 6. Mutant analysis for the putative remorin. The homozygous remorin UniformMu mutant lines are significantly more susceptible than the homozygous wild-type lines. Note: the line with a wild-type allele was W22, not wild-type segregants from the family.

Table 1. Fine-mapping markers. KASPar markers used for fine-mapping study. Physical positions are based on AGP_V2.

Marker id	Locus name	Position	Forward primer 1	Forward primer 2	Reverse primer
snp_01_0153	PZA02372.1	6,220,547	GAAGGTGACCAAGTTCATGCTTGTGCAGGCGATGCTGGTCA	GAAGGTCGGAGTCAACGGATTTGTGCAGGCGATGCTGGTCG	TACAGGGGCAGGTCGGAGGA
snp_01_0154	PZA03093.10	8,075,572	GAAGGTGACCAAGTTCATGCTGCGGGAGCATTGATAGGCTTC	GAAGGTCGGAGTCAACGGATTGCGGGAGCATTGATAGGCTTG	GACGCGGACAAGTTCGTTGAGT
snp_01_0155	AY105791	8,510,027	GAAGGTGACCAAGTTCATGCTCAGCAGGAAGCTGCTGAGGAA	GAAGGTCGGAGTCAACGGATTCAGCAGGAAGCTGCTGAGGAT	GGGCACCGCCATGTCCCT
snp_01_0156	PZA00731.7	9,300,541	GAAGGTGACCAAGTTCATGCTGTGGAGGTCAGAAACAAAGAGTC	GAAGGTCGGAGTCAACGGATTAGTGGAGGTCAGAAACAAAGAGTT	ATGAGCATCAACAGCACTGAA
snp_01_0063	ss196517836	15,071,288	GAAGGTGACCAAGTTCATGCTCAAGGGCTTCAAGAACTTGCCCA	GAAGGTCGGAGTCAACGGATTAAGGGCTTCAAGAACTTGCCCC	TTACGAGGAGCACGGACGGCAT
snp_01_0031	PZA02393.2	16,581,396	GAAGGTGACCAAGTTCATGCTCATCTCCATATGAATGAGTGCATC	GAAGGTCGGAGTCAACGGATTCATCTCCATATGAATGAGTGCATT	CCGTAGAAAAAAATGTGGAAAAACAAYGAA
snp_01_0065	ss196517546	17,746,383	GAAGGTGACCAAGTTCATGCTAAGAGGTGCCAGAGCGAGTGT	GAAGGTCGGAGTCAACGGATTGAGGTGCCAGAGCGAGTGC	GTCATTACATGAGGTTTGAGGAGAGTTT

snp_01_0066	ss196516974	18,444,560	GAAGGTGACCAAGTTC ATGCTAATTGAACAAG CCATACGGGAAGATA	GAAGGTCGGAGT CAACGGATTGAA CAAGCCATACGG GAAGATG	GGTCATGCTAGTTT GTGGTTGACGAT
snp_01_0067	ss196523722	19,679,687	GAAGGTGACCAAGTTC ATGCTAGCTAGCTCCTC GGGAAGAGA	GAAGGTCGGAGT CAACGGATTGCT AGCTCCTCGGGA AGAGG	GCGCCGGCCTGTGC CTGTT
snp_01_0071	ss228815856	20,110,551	GAAGGTGACCAAGTTC ATGCTATGCCGGGGTTT CGGGAGG	GAAGGTCGGAGT CAACGGATTCAT GCCGGGGTTTCG GGAGA	CGCGGCCGAACCGG CGAA
snp_01_0029	chr1|21461479	21,472,299	GAAGGTGACCAAGTTC ATGCTGCTGTTTCGCAT TTGATTCGAC	GAAGGTCGGAGT CAACGGATTGCT GCTGTTTCGCATT TGATTCGAT	TGAACAAGCTATCA GCGCCGGTTA
snp_01_0030	chr1|21514247	21,525,088	GAAGGTGACCAAGTTC ATGCTGTCGCTAAAAC CTGACCAGGG	GAAGGTCGGAGT CAACGGATTGTC GCTAAAACCTGA CCAGGA	CTCGTCTGTGCGCA GCGGCA
snp_01_0072	ss196507791	22,379,568	GAAGGTGACCAAGTTC ATGCTTCTATTCTGGTC AATCTTTGCA	GAAGGTCGGAGT CAACGGATTGCTT CTATTCTGGTCAA TCTTTGCC	CATTTTCTAACAATC GAGTCCACAACCTT
snp_01_0033	PZA02487.1	22,595,964	GAAGGTGACCAAGTTC ATGCTCCAAAATGTGA AGAACTTCCAAAAGGT T	GAAGGTCGGAGT CAACGGATTCAA AATGTGAAGAAC TTCCAAAAGGTC	ACCCATCTGCTGGC GAATAGTAGTT
snp_01_0068	ss196526861	24,225,835	GAAGGTGACCAAGTTC ATGCTTGTTCTTGCCAC	GAAGGTCGGAGT CAACGGATTGTTC	AGAACGTAGTACGT AGAGTTACACTACT

			AAACGGTACTTTTT	TTGCCACAAACG	T
				GTACTTTTG	
				GAAGGTCGGAGT	
			GAAGGTGACCAAGTTC	CAACGGATTATG	GCTGCATTCTGCCA
			ATGCTATGATCTTGCTA	ATCTTGCTACTCA	AAGTGTTAAGATAA
snp_01_0073	PZA02921.4	24,940,817	CTCAGGTGCATC	GGTGCATG	T
				GAAGGTCGGAGT	
			GAAGGTGACCAAGTTC	CAACGGATTACCT	
			ATGCTACCTTCGTTAGT	TCGTTAGTTCTAG	CCAAAGGCTTTTGC
snp_01_0129	ss228821000	25,156,553	TCTAGTTGTATAAGG	TTGTATAAGT	AGCAATCTCGAA
				GAAGGTCGGAGT	
			GAAGGTGACCAAGTTC	CAACGGATTGGG	
			ATGCTGGGGCCCGAGG	GCCCGAGGTGGG	GGTTTCGCGCCCGT
snp_01_0131	ss228821166	25,370,872	TGGGA	G	GCGCTA
				GAAGGTCGGAGT	
			GAAGGTGACCAAGTTC	CAACGGATTACG	
			ATGCTACGCAACAGAT	CAACAGATGGAA	CATGCCTTATTGCTT
snp_01_0140	ss228821181	25,371,937	GGAAAATCACATAAG	AATCACATAAC	TTGCAGTGTTTGTT
				GAAGGTCGGAGT	
			GAAGGTGACCAAGTTC	CAACGGATTGTC	
			ATGCTCGTCACTGCAA	ACTGCAACTTTCG	GCCTAACATTTACT
snp_01_0141	ss228821208	25,374,304	CTTTCGGCAGT	GCAGC	GCAGCGTGCAT
				GAAGGTCGGAGT	
			GAAGGTGACCAAGTTC	CAACGGATTCGG	
			ATGCTCGGCGTCGTAG	CGTCGTAGTCTCC	CTCACATGAGCCAC
snp_01_0142	ss228821319	25,377,803	TCTCCGTAG	GTAC	AACAAGCAGTA
				GAAGGTCGGAGT	
			GAAGGTGACCAAGTTC	CAACGGATTAGTT	CAGTCAAACTGCAG
			ATGCTAGTTGAACCATT	GAACCATTTGTCT	CCTTCATCAAAAAT
snp_01_0143	ss228821373	25,387,185	TGTCTGCTCATGC	GCTCATGA	AA

snp_01_0145	ss228821424	25,399,986	GAAGGTGACCAAGTTCATGCTGCAGCACATTGCTACGG	GAAGGTCGGAGTCAACGGATTCATGCTGCAGCACATTGCTACGA	GCCTTTCACGAGGCTGGTCCTA
snp_01_0074	ss196505525	25,411,250	GAAGGTGACCAAGTTCATGCTGAGAAGTGCAACTACAATCAGTGC	GAAGGTCGGAGTCAACGGATTGGAGAAGTGCAACTACAATCAGTGT	GGAAAACAAACATAAAGCATATACCAGATT
snp_01_0146	ss228821481	25,414,726	GAAGGTGACCAAGTTCATGCTCACCAACATCCATCGCCTTCG	GAAGGTCGGAGTCAACGGATTGCACCAACATCCATCGCCTTCA	CAAGAACAAGCCTTGGCCTCAAACTT
snp_01_0147	ss228821512	25,416,567	GAAGGTGACCAAGTTCATGCTGAGTATTGAATCAAGGTTTCCTTTTAAGAT	GAAGGTCGGAGTCAACGGATTGAGTATTGAATCAAGGTTTCCTTTTAAGAA	GGTATTTTTGCATGCAGAATTTGTACTGTA
snp_01_0150	ss228821595	25,434,036	GAAGGTGACCAAGTTCATGCTGTCACCAAATCACGTGATTGGCT	GAAGGTCGGAGTCAACGGATTGTCACCAAATCACGTGATTGGCC	CCGGTGCCTCCCAGAAAAGGAA
snp_01_0151	ss228821616	25,451,415	GAAGGTGACCAAGTTCATGCTAGAAAATGTTCAGCAGCACCCATGT	GAAGGTCGGAGTCAACGGATTAGAAAATGTTCAGCAGCACCCATGA	GGAACACAAACTCAGCATGCACCAA
snp_01_0152	ss228821639	25,452,796	GAAGGTGACCAAGTTCATGCTGATAAACCGATCGACTAGTCACC	GAAGGTCGGAGTCAACGGATTCGATAAACCGATCGACTAGTCACT	CGTCTAGGCCCCGGTGACTATT
snp_01_0132	ss228821641	25,452,934	GAAGGTGACCAAGTTC	GAAGGTCGGAGT	GAAAACCACTCCTA

			ATGCTGTATAGAGCTATTGGGCCTTATGG	CAACGGATTGTATAGAGCTATTGGGCCTTATGA	AATGGACCGGTT
snp_01_0128	ss228821609	25,624,562	GAAGGTGACCAAGTTCATGCTGAGCATCCTTGCTGCAATGCC	GAAGGTCGGAGTCAACGGATTCGAGCATCCTTGCTGCAATGCT	CATCGACATGGATATCGCCATGTCAA
snp_01_0069	ss196501159	26,197,833	GAAGGTGACCAAGTTCATGCTAGGAAGAAGAGGAAGTGGGATCAA	GAAGGTCGGAGTCAACGGATTGGAAGAAGAGGAAGTGGGATCAG	GTCACCGCCGCGGAGACCAA
snp_01_0105	Rik4080	26,199,729	GAAGGTGACCAAGTTCATGCTATCGGTCCCTTTCTCCTCTAGC	GAAGGTCGGAGTCAACGGATTATATCGGTCCCTTTCTCCTCTAGT	GGTAAATTTGCTCATGCTACGGAGATA
snp_01_0106	Rik5245	26,200,894	GAAGGTGACCAAGTTCATGCTGGTGTCGACACCTCAGGAACA	GAAGGTCGGAGTCAACGGATTGGTGTCGACACCTCAGGAACG	TAACACATTGGGCCCTACAATATAGTGAA
snp_01_0107	Rik5416	26,201,065	GAAGGTGACCAAGTTCATGCTCAGTAACATGGCCTATCCTATTCCA	GAAGGTCGGAGTCAACGGATTAGTAACATGGCCTATCCTATTCCC	GGATAAATGTCCCCATAACCACTGTAAAA
snp_01_0108	Rik7293	26,202,942	GAAGGTGACCAAGTTCATGCTGGATCAAATGGAATGCTTCCGC	GAAGGTCGGAGTCAACGGATTGGATCAAATGGAATGCTTCCGG	AGTGGATATAGAGAGATGTGATGGCATAT
snp_01_0109	Rik7294	26,202,942	GAAGGTGACCAAGTTCATGCTGTGATGGCATATCCGCTTCTTCTT	GAAGGTCGGAGTCAACGGATTGATGGCATATCCGCTT	CCGAAGAAAGTTCACCCTGGATCAA

snp_01_0032	PZB01957.1	26,202,944	GAAGGTGACCAAGTTCATGCTGTGATGGCATATCCGCTTCTTCTT	CTTCTGGAAGGTCGGAGTCAACGGATTGATGGCATATCCGCTTCTTCTG	CTCCGAAGAAAGTTCACCCTGGAT
snp_01_0110	Rik7724	26,203,373	GAAGGTGACCAAGTTCATGCTATTATGGAGATGACGACGATGACGA	GAAGGTCGGAGTCAACGGATTATGGAGATGACGACGATGACGG	TGAACCAGGAGTTGGATTGCCCTTA
snp_01_0111	Rik7748	26,203,397	GAAGGTGACCAAGTTCATGCTGGAGTTGGATTGCCCTTAGGTG	GAAGGTCGGAGTCAACGGATTAGGAGTTGGATTGCCCTTAGGTA	GAGATGACGACGATGACGRCGATA
snp_01_0112	Rik7965	26,203,614	GAAGGTGACCAAGTTCATGCTTCTTTGAACTTGTATTCATTCTGGCC	GAAGGTCGGAGTCAACGGATTCTTTGAACTTGTATTCATTCTGGCG	TCGTATCTGAACCAAAACAACAAACGGTT
snp_01_0113	Rik8057	26,203,706	GAAGGTGACCAAGTTCATGCTTGGTTGCTGAACTCGTAGCATTTTAA	GAAGGTCGGAGTCAACGGATTGGTTGCTGAACTCGTAGCATTTTAT	ATAGGCTCTAACAATTAYATAATACCGACA
snp_01_0114	Rik8070	26,203,719	GAAGGTGACCAAGTTCATGCTGATACAAAATTGACATAGGCTCTAACAATTAT	GAAGGTCGGAGTCAACGGATTACAAAATTGACATAGGCTCTAACAATTAC	CGTTGGTTGCTGAACTCGTAGCATT
snp_01_0075	PZA03004.2	26,435,043	GAAGGTGACCAAGTTCATGCTGAACACATGGGGCCTTGTA	GAAGGTCGGAGTCAACGGATTCTGAACACATGGGGCCTTGTG	GGGAATAGCATCAARGCCTCAAGGT

snp_01_0076	ss196526176	26,911,913	GAAGGTGACCAAGTTCATGCTCGGGTGCACAAGAATTTCATCTATCA	GAAGGTCGGAGTCAACGGATTGGGTGCACAAGAATTTCATCTATCG	CTTTCTGTATATGTGTATGCACACAGACAA
snp_01_0028	chr1|27735302	27,630,429	GAAGGTGACCAAGTTCATGCTCCGCCACTTGACAGCAAAAAGAATT	GAAGGTCGGAGTCAACGGATTCGCCACTTGACAGCAAAAAGAATC	CCATGCAGTCTTTGCAGAATTAGGCAT
snp_01_0027	chr1|28209839	28,067,592	GAAGGTGACCAAGTTCATGCTACGTAGTAACTAATCACGTCTTTATTCTA	GAAGGTCGGAGTCAACGGATTCGTAGTAACTAATCACGTCTTTATTCTG	GAATTAACGAGGGTGTTTGGAACCTATAT
snp_01_0034	PZB02058.1	28,421,841	GAAGGTGACCAAGTTCATGCTCATCAAGTTCAAAAGGAGGGCAG	GAAGGTCGGAGTCAACGGATTGCATCAAGTTCAAAAGGAGGGCAA	TAAGCACACATTCCACCTGATGTGAAATT
snp_01_0026	chr1|29925693	29,820,814	GAAGGTGACCAAGTTCATGCTCGGTGAGTGCGTGTGCGTAT	GAAGGTCGGAGTCAACGGATTCGGTGAGTGCGTGTGCGTAC	AGAGGAAGATGGTGCACCGGCA
snp_01_0025	chr1|29961168	29,856,293	GAAGGTGACCAAGTTCATGCTGGAGATTAGAGAGAGGAGAACGT	GAAGGTCGGAGTCAACGGATTGGAGATTAGAGAGAGGAGAACGC	CGAGCTCGGCCGTTGGCCAT
snp_01_0070	ss196528084	31,860,782	GAAGGTGACCAAGTTCATGCTAGAACAAGCTCCTTCATTCTACTAGT	GAAGGTCGGAGTCAACGGATTGAACAAGCTCCTTCATTCTACTAGC	GAGGAAGTCGAAAAGCTAACTGTACAAAA
snp_01_0035	PZA01455.1	34,317,549	GAAGGTGACCAAGTTCATGCTGTTGAGGTCGA	GAAGGTCGGAGTCAACGGATTGTTG	GGAGCACAAAAACAAAAGCAACACGAAT

	GGTGACCA	AGGTCGAGGTGA CCG	A

Table 2. Primers. qPCR, mutant analysis, and sequencing primers.

Name	Gene/ insertion	Forward primer	Reverse primer	Probe
GRMZM2G070442_487	GRMZM2G070442	GGTCCTGCAAGAGGAACTACAAG	CGGGTAGATCACGTCGTTCTC	CGGGTACGTGTGGAAC
GRMZM2G523621_54	GRMZM2G523621	ACGCGAAGAGGAGGCTGAT	TGCGGCTGAGGAGCTTTG	AGGATGTGAGGATGCG
mut_01_0019	mu1051418	CTTGACACTACTGGGCCGAT	TGGCCGACGCGAATAGTAAC	-
mut_01_0022	mu1046469	TGTTTACACTACCTGGCCCCT	GCAGGGTTCGGTTTTCTGGA	-
mut_01_0023	mu1058569	ACGGATCAGACAGGATTGAGC	CGGCCCATCCGCGAATAATA	-
TIR6	-	AGAGAAGCCAACCTCYATTTCGTC	GCCAWCGC	-
seq_01_0001	*rik*	AACTACCGGTTTCGAAGGGCCCA	CCACAGCCAATGCAAACTCCGATTT	-
seq_01_0002	*rik*	AGTTGGATTGTCAGATTGGCATCAT	GGGTGGTGGTAGCATCTTAGTGGA	-
seq_01_0003	*rik*	ATGCTTCCGCAAGAAGAAGCGGA	AGCAATGTCGTGACCTTAGGCTG	-
seq_01_0004	*rik*	TCGACGTTGGTTGCTGAACTC	TCTTGCCATGTCGATTCTTGACTC	-
seq_01_0005	*rik*	ACATTCAGTGGAAGCTCCAAGCA	TGCACCAAGGCAGCAAGCAT	-
seq_01_0006	*rik*	TCTTCACATTCAGTGGAAGCTCCA	TCGCCGTCATCGTCGTCATC	-
seq_01_0007	*rik*	AACGTGCTCGATCGTTCGTTCTCT	TCCACAGTACCTCTCGGAGCCAA	-
seq_01_0008	*rik*	GTTCCGTATTCATTGCCGCCCCA	GCCTCCCTGGCCCCCTGTTTAAT	-

		GGGCCAGGGA GGCTAGAAAG	TGAGGCACT AAAAGGAC	
seq_01_0009	*rik*	GAA	GAACAGCC CCAAGAAG	-
		CTGCTGAGCGC	GTGACAATG	
seq_01_0010	*rik*	ATTAAGGCAGT ACCTTTGCATC	CGGATGC ACAGGGAA	-
		CGCATTGTCAC	GGAAGGGG	
seq_01_0011	*rik*	C	TTGCCA	-

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CHAPTER 4

TESTING THE HYPOTHESIS: VALIDATION OF ASSOCIATION MAPPING FOR NORTHERN LEAF BLIGHT IN MAIZE[4]

Abstract

Genome-wide association studies (GWAS) detect significant genotype-phenotype relationships, which can be used to identify candidate genes. These candidate genes must be subsequently validated. We utilized nested association mapping (NAM) in maize to identify genes putatively associated with disease resistance. The NAM population consists of 5,000 recombinant inbred lines derived from 25 founder lines (Yu *et al*., 2008). The GWAS using northern leaf blight (NLB) phenotypes and HapMap2 genotypes on the NAM (NLB; Poland *et al*., 2011, Chia *et al*., 2012) served as a basis for an extensive list of candidate genes. To determine the proportion of significant associations that could be confirmed through mutant phenotypes, we inoculated mutant maize lines corresponding to >100 genes identified by GWAS and measured their quantitative NLB phenotypes. Lines were identified from the UniformMu project that carried insertions within or adjacent to the GWAS-based candidate genes (Settles *et al*., 2007; McCarty *et al*., 2013). We phenotyped 123 UniformMu families to find lines with significant differences in disease phenotype. Among them, approximately 10% of the families showed significant differences in disease phenotypes. Most of the significant lines were selected for insertions in genes within joint linkage mapping QTL. Approximately 75% of those significant lines

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corresponded to genes within joint linkage mapping NLB QTL (Poland *et al*., 2011). Approximately 37% were significantly different than the background inbred line 'W22'.

Introduction

A number of genome-wide association studies (GWAS) have been conducted to understand the genetic basis of complex traits, including disease resistance in maize. A large number of candidate genes have been suggested in maize through association mapping across various populations (Poland *et al*., 2011; Chia *et al*., 2012; Van Inghelandt *et al*., 2012; Schaefer and Bernardo 2013). Little has been done to confirm significant associations across independent germplasm sets, or to substantiate the role of candidate genes in pathogenesis through other methods, including functional and mutant studies.

There are various reasons to infer that genome-wide association studies are prone to type 1 error. Polymorphisms not underlying natural variation might be significant in an association mapping study because they are in linkage disequilibrium with the causative polymorphism (Platt *et al*., 2010). Population structure and epistasis can further contribute to false positives (Platt *et al*., 2010). Other factors that may contribute to the difficulty of conducting GWAS in maize include the prevalence of low-frequency alleles, presence/absence variation, and the generally rapid breakdown of LD, which requires high-density genotypic coverage. Additional support must be provided to validate significant GWAS associations.

To test the reliability of association mapping, mapping has been performed for

traits where the underlying genes are known and previous association mapping results reviewed with updated methods. In the case of kernel color in maize, the strongest association from the USDA-ARS RRIS collection was within *Y1*, a gene involved in carotenoid pigmentation in the endosperm (Romay *et al.,* 2013). However, from the same study, a lower frequency trait, namely sweet versus starchy corn, significant polymorphisms defined a 14-Mb interval around *Su1* (Romay *et al.,* 2013). In other instances of further reanalysis of data, such as for the role of *Dwarf8* in flowering time in maize, errors associated with association mapping have been unveiled (Larsson *et al.,* 2013). From this study, it is clear that population structure needs to be properly accounted for in the statistical models used in association mapping.

One method of validating candidate genes is through the evaluation of mutant alleles to determine their effect on the phenotype of interest. The relationship between QTL and mutants has been explored in other species and for other traits. There are a number of studies in *Drosophila* where mutant populations have been screened for extreme phenotypes (Norga *et al.,* 2003; Harbison *et al.,* 2004; Magwire *et al.,* 2010). Overlap is seen in these studies between mutants and QTL (Norga *et al.,* 2003), and complementation tests with mutants have verified candidate genes underlying QTL (Edwards and Mackay 2009).

In crop species, the relationships between mutants and QTL have varied. Results vary for the co-localization of QTL with genes in pathways known to be important to or mutants known to have an effect on the trait (Weng *et al.,* 2011; Peiffer *et al.,* 2013; Peiffer *et al.,* 2014). Authors hypothesized that this may be due to a lack of natural variation in these genes or to negative fitness effects (Peiffer *et al.,*

2013). In rice, *ART1* was first described as involved in aluminum tolerance in rice based on a mutant study (Yamaji *et al*., 2009; Xia *et al*., 2010). Subsequently, a QTL study by Famoso *et al*. (2011) identified *ART1* to be within the target region of an aluminum tolerance QTL.

The objective of this study was to validate genes implicated by association mapping for resistance against northern leaf blight (NLB) in maize. This was achieved by identifying insertional mutant families with an effect on disease phenotype and by examining significant mutant-phenotype interactions. Lines significantly differing from the mutant background line 'W22' were identified. Lines showing significant within-family variation were also considered. Lines were then genotyped and genotype-phenotype interactions examined. Using this approach we explored the reliability of GWAS and examined the relationship between QTL and mutants.

Materials and methods

Identification of genes implicated by genome-wide nested association mapping

A list of genome-wide nested association mapping results with BPP>1 was obtained from Chia *et al*. (2012). This analysis was completed using 55 million polymorphisms included in the HapMapV2 dataset (Chia *et al*., 2012), as well as the 1 million polymorphisms included in HapMapV1 dataset (Gore *et al*., 2009). This list included 1,332 associations with BPP>1. Physical position coordinates of this dataset were based on B73 AGPv1 reference genome coordinates (Schnable *et al*., 2009). Galaxy was used to identify genes and domains associated with GWA results (Goecks *et al*., 2010). AGP_v1 gene models were downloaded from http://www.gramene.org

(Youens-Clark *et al*., 2011). Positions of the associations were anchored to AGPv1 and associations were classified as either genic or intergenic using Galaxy (Goecks *et al*., 2010). If associations were genic, the gene id was added to the candidate gene list. If the association was intergenic, the gene ids of the first gene to the left and first gene to the right were added to the candidate gene list. Interpro domains for each candidate gene were identified by joining the candidate gene list and Interpro domains for all genes with Galaxy (Goecks *et al*., 2010).

Identification and selection of UniformMu lines carrying insertions within or adjacent to genes of interest

A list of all available F_4 UniformMu lines, which included the genes interrupted by a *Mu* transposon in each line, was downloaded from maizegdb.org (Andorf *et al*., 2010). The candidate gene list was cross-referenced to this list using Galaxy (Goecks *et al*., 2010). If multiple UniformMu lines were available for a given gene, only one UniformMu line was selected for each gene, with the exception of associations with BPP>15 that were located within intervals based on previous QTL mapping studies. Also included were genes that were also implicated by southern leaf blight (SLB) GWAS (Chia *et al*., 2012) and those that were within regions of interest, namely the fine-mapping intervals located in maize bins 1.02, 1.06, or 8.06.

Genotyping

CTAB DNA was extracted as described by Doyle and Dickson (1987) and Chung *et al*. (2010b). Primers were designed based on sequences upstream and downstream of

the insertion to amplify a product of approximately 500 bp using Primer3 (Untergasser *et al*., 2012). TIR6 and TIR8 were used to amplify the transposon (Settles *et al*., 2004). Primers were obtained from IDT (Coralville, Iowa, USA).

Phenotypic evaluation

Northern leaf blight

NLB evaluations were conducted at Cornell's Robert Musgrave Research Farm in Aurora, NY in 2013. An incomplete block design was used, whereby each block was composed of a UniformMu family and a control wild-type W22. Each family consisted of a number of rows derived from a single stock of 15 kernels. Two replications were included for the large-scale screen, while three replications were included for high-interest mutant families that fell within the 1.06 fine-mapping interval (Jamann *et al*., 2014). NLB inoculations were performed as described by Chung *et al*. (2010a) using *S. turcica* race 1 isolate StNY001. Disease was assessed weekly on a row basis starting two weeks prior to flowering using a 0-100 percentage scale with an increments of one. Diseased leaf area (DLA) ratings were then used to calculate area under the disease progress curve (Wilcoxson *et al*., 1974; Chung *et al*., 2010a).

Flowering time

Dates were recorded when half of the plants within a row were shedding pollen. This date was then subtracted from the planting date (May 17, 2013) to calculate the days to anthesis.

Statistical analysis

Each family was derived from a single UniformMu stock and each family was analyzed individually for within-family differences. Stock lines was increased in winter nursery and multiple rows derived from each stock were evaluated in the field in 2013. Each family was analyzed to identify significant differences among rows, or within families. In order to identify families with significant within-family variation, a mixed model was run in JMP 9.0 (Cary, NC, USA) with AUDPC as the response and replication as a random factor. R was used to create box plots and histograms (R Core Development Team 2013).

As a complimentary approach, AUDPC values were subtracted from control W22 lines within blocks and confidence intervals (95%) were computed based on the deviations of rows from the control line (W22) row to identify phenotypic extremes. Subtracting disease values from W22 was done to account for the variation in disease across the field. Secondly, a Dunnett's test was performed to identify lines that differed significantly from W22. These two criteria were used to identify families that significantly differed from W22 (Magwire *et al*., 2010).

Results

Candidate gene identification

A total of 1,588 genes were identified. Of these, 243 SNPs were located within genes and the rest were implicated by an intergenic association. A total of 606 of the genes implicated by genome-wide nested association mapping had corresponding

UniformMu lines associated with them. In order to reduce the number of UniformMu lines for phenotypic analysis, criteria were imposed. For most of the genome, only genes with associations with BPP>15 were taken forward. Genes are shown in Table 1. For intervals of particular interest (1.02, 1.06, and 8.06) all implicated UniformMu lines were included, as well as any lines associated with genes implicated by both the NLB and SLB GWAS. This reduced the UniformMu stock lines to n=123. The UniformMu lines have many insertions and may be heterozygous for insertions of interest. The lines were ordered from the stock center and then self-pollinated in winter nursery to increase seed and to generate families including homozygous insertion lines. The resulting F_6 progeny were scored for NLB severity.

Families showing significant differences

Upon analysis of phenotypic data within families, we found 12 of 123 (9.8%) families to be significant, as shown in Table 2 and Fig. 1. Of these, five were in the 8.06 interval of interest. Furthermore, six were also implicated by association analysis of SLB. Nine of these genes fell within NAM joint linkage mapping QTL (Poland *et al.,* 2011). Interestingly, no associations among the 12 had BPP values greater than 50 and about half had BPP values of 1.

Families with significantly different phenotypes than W22

The Dunnett's test revealed 46 families (37.4%) that were significantly different than W22, as shown in Table 3. Additionally six families were identified from confidence interval calculations, five of which were identified by the Dunnett's test. Combined,

47 lines were identified as significantly different than W22, or 38.2% of the total lines tested. Lines are shown in Table 3.

Discussion

In this study, we found that only 9.8% of UniformMu lines had significant differences within families, and 38.2% of families were significantly different than W22. The families not identified by these two analyses could be due to false positives from the association analysis, small effects of the QTL, or a non-functional copy in wild-type W22. Additional genotyping is needed to establish significant genotype-phenotype segregation. The list of families with significant genotype-phenotype segregation will likely be different than the within- or among- family analyses.

Many of the significant within-family families had insertions in genes within joint linkage mapping QTL, as shown in Figure 1. Further testing is needed to determine whether this was due to biases of the experimental design, or whether GWAS associations within joint linkage QTL are more likely to be real.

This data suggests that there may be a basis for pleiotropic multiple disease resistance. Half of the significant families from the within-family analysis were also implicated by association analysis for SLB. These families should be tested for SLB. Linkage was believed to play a large role in the instances of multiple disease resistance that have been observed as opposed to pleiotropy (Wallace *et al.*, 2014). This data suggests that there may be instances of single genes having effects on resistance to multiple diseases.

In this study, the genes immediately upstream and downstream of an

association were considered the candidate genes for the intergenic polymorphism. Another approach used in association studies is to consider all of the genes that are in LD with the significant hits in this region (Chen *et al*., 2012). This approach would provide a more accurate representation of the genes implicated by association mapping, but would introduce additional candidates.

Here we present evidence to support candidate genes implicated by GWAS. Each family had many insertions and lines must be genotyped for insertions of interest. Additional phenotyping is also necessary. Phenotypes here are based on one season of data. A genotype-phenotype association is needed to substantiate the hypotheses established here.

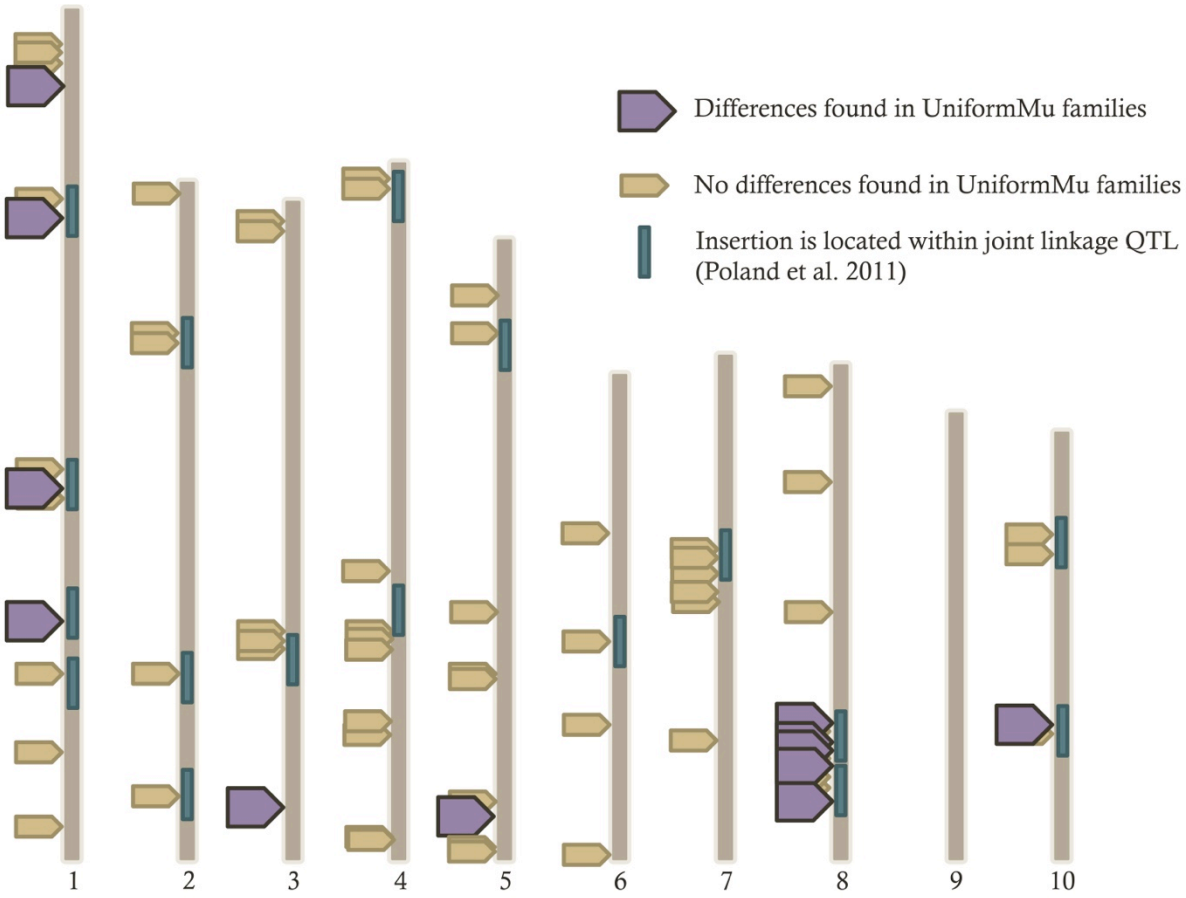

Figure 1. Insertion map. This figure shows the chromosomal location of insertions tested for NLB and whether they lie within NLB joint linkage QTL. Those insertions denoted in purple have mutants with significantly different NLB phenotypes, while no significant phenotype was found in mutant lines shown in gold.

Table 1. Genes implicated by Chia *et al.* (2012) for NLB resistance. Genome-wide association mapping results for NLB from Chia *et al.* (2012) were used to identify genes containing significant associations or adjacent to significant associations if implicated polymorphism was intergenic. Genes implicated by a bootstrap posterior probability greater than 15 are shown.

BPP	Allele	Interpro Domain
78	T/C	Serine-threonine/tyrosine-protein kinase
78	T/C	Uncharacterized gene
63	G/C	F-box domain, cyclin-like
47	CNV+	Disease resistance protein
47	CNV+	Disease resistance protein
44	CNV-	Serine-threonine/tyrosine-protein kinase
44	CNV-	Zinc finger, A20-type
43	A/G	Uncharacterized gene
41	A/G	Uncharacterized gene
40	T/C	Uncharacterized gene
40	T/C	Spermine synthase
31	A/G	NAD-dependent epimerase/dehydratase
28	C/T	Bromodomain
28	C/T	Uncharacterized gene
25	G/A	Exoribonuclease, phosphorolytic domain 2
25	G/A	Isopenicillin N synthase
25	C/T	Ras GTPase
25	T/C	Immunodeficiency virus transactivating regulatory protein (Tat)
25	T/C	Uncharacterized gene
23	A/G	Rad21/Rec8 like protein, C-terminal
23	A/G	Uncharacterized gene
21	C/T	DNA mismatch repair protein MutS, C-terminal
19	T/C	Transferase
19	T/C	EGF-type aspartate/asparagine hydroxylation site
19	C/A	Protein of unknown function DUF125, transmembrane
18	G/A	Uncharacterized gene
17	T/G	Serine-threonine/tyrosine-protein kinase
16	C/A	Per1-like
16	C/A	Cytochrome P450
16	G/A	Like-Sm ribonucleoprotein (LSM) domain
16	G/A	Uncharacterized protein family UPF0497, trans-membrane plant subgroup
15	A/G	Prenyltransferase/squalene oxidase
15	A/G	Serine-threonine/tyrosine-protein kinase
15	C/T	Uncharacterized gene
15	C/T	Zinc finger, B-box
15	G/C	Cryptochrome/DNA photolyase, class 1
15	C/T	Lipase, GDSL
15	C/T	Uncharacterized gene

Table 2. Families with significant within-family variation for NLB. To identify families of high priority for genotyping, families were analyzed for significant within-family differences. These families are being genotyped to analyze co-segregation of phenotype and genotype.

Family	Gene	Chromosome	Joint Linkage QTL (Poland *et al.,* 2011)	GWAS Disease
UFMu-06756	Protein of unknown function	Chr1	Inside	NLB, SLB
	Zinc-finger	Chr1	Inside	NLB
UFMu-07364	Major intrinsic protein	Chr1	Inside	NLB, SLB
UFMu-01980	Adenylate kinase	Chr3	Outside	NLB, SLB
UFMu-01984	Armadillo	Chr5	Outside	NLB, SLB
UFMu-06512	C2 calcium-dependent membrane targeting	Chr8	Outside	NLB
UFMu-06007	WD40 repeat	Chr8	Inside	NLB
UFMu-05423	Target SNARE coiled-coil domain	Chr8	Inside	NLB, SLB
UFMu-05156	MADF domain	Chr8	Inside	NLB
UFMu-03115	Lipase, GDSL	Chr8	Inside	NLB
UFMu-02602	Kinesin, motor domain	Chr8	Inside	NLB
UFMu-03522	Lipase, GDSL	Chr10	Inside	NLB, SLB

Table 3. UniformMu lines significantly different than W22 for NLB. Lines (n=47) that significantly differed from W22 based on either a Dunnett's test or confidence intervals.

Significant lines	Method
UFMu-00652	Dunnett's
UFMu-00841	Dunnett's
UFMu-00895	Dunnett's
UFMu-01040	Dunnett's
UfMu-01180	Dunnett's
UFMu-01373	Dunnett's
UFMu-01469	Dunnett's
UFMu-01716	95% Confidence interval; Dunnett's
UFMu-01882	Dunnett's
UfMu-01990	Dunnett's
UFMu-01998	Dunnett's
UFMu-02492	Dunnett's
UFMu-02596	Dunnett's
UFMu-02632	Dunnett's
UFMu-02693	95% Confidence interval
UFMu-02750	95% Confidence interval; Dunnett's
UFMu-02776	Dunnett's
UFMu-03016	Dunnett's
UFMu-04211	Dunnett's
UFMu-04414	Dunnett's
UFMu-04443	Dunnett's
UFMu-04549	Dunnett's
UFMu-05553	Dunnett's
UFMu-05759	Dunnett's
UFMu-06342	Dunnett's
UFMu-06455	Dunnett's
UFMu-06511	Dunnett's
UFMu-06514	Dunnett's
UFMu-06550	Dunnett's
UFMu-06564	Dunnett's
UFMu-06572	Dunnett's
UFMu-06583	Dunnett's
UFMu-06591	Dunnett's
UFMu-06637	Dunnett's
UFMu-06682	Dunnett's
UFMu-06715	Dunnett's

UFMu-07245	Dunnett's
UFMu-07380	Dunnett's
UFMu-07408	95% Confidence interval; Dunnett's
UFMu-07882	Dunnett's
UFMu-07888	Dunnett's
UFMu-07985	Dunnett's
UFMu-08013	Dunnett's
UFMu-08041	Dunnett's
UFMu-08088	Dunnett's
UFMu-08146	Dunnett's
UFMu-08159	Dunnett's

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CHAPTER 5

CONCLUSIONS

Quantitative disease resistance (QDR) is a complex phenomenon and determining the genes and mechanisms underlying it is difficult. Many genes may have small effects on resistance phenotypes. The maize genome is plastic and often times the reference genome is not the resistance donor. Advances have been made in offering glimpses into the maize pan-genome, but high-quality assembled, annotated sequence information is available only for the reference line. Despite these difficulties, I have identified candidate genes for resistance to northern leaf blight (NLB) and Stewart's wilt. I have validated *pan1* as a susceptibility gene for both diseases and have also presented strong evidence for the putative remorin gene and the putative F-box gene as resistance-related genes. This dissertation touches on a number of topics relating to QDR, including the genetic architecture and basis of QDR and the pleiotropic nature of disease resistance in maize.

Genetic architecture of disease resistance

The genetic architecture of disease resistance in maize is even more complex than previously thought. Poland *et al*., (2011) and Kump *et al*., (2011) identified 29 QTL for northern leaf blight and 32 QTL for southern leaf blight, respectively. The findings presented in this dissertation suggest that not only do multiple genes underlie each locus, but structural variation also plays a role in disease resistance. This complexity

makes it exceedingly difficult to identify and confirm genes associated with resistance and a multi-faceted approach is needed to address the complexity of disease resistance.

The complex genetic architecture also reflects the long co-evolution of maize and the microbes that associate with it. Wallace *et al*. (2013) presented the hypothesis that traits that have been selected upon for a long time horizon have more QTL of smaller effects. That is what we have found for the interaction between maize and *S. turcica* with many loci contributing to the interaction. This contrasts with gray leaf spot, for example, where the jump to maize pathogenicity is more recent and there are a smaller number of QTL (Benson 2013). Combining multiple loci may provide larger effect, more durable resistance. A better understanding of the mechanisms of resistance and their deployment could improve breeding for disease resistance.

For disease resistance, as well as for other traits, that there are regions of the genome that contain clusters of QTL, including maize bin 1.06. Bin 1.06 is important not only for disease, but also other agronomic traits, and offers some insight into the relationship between genome structure and trait-associated variation. It is a tantalizing hypothesis that there is a haplotype associated with variation for multiple traits that is retained through selection and genome structure at the region that favors low recombination. A next step in addressing this hypothesis might be to evaluate the fine-mapping population for other traits to examine the co-localization of the QTL.

This research suggests that there may be a role for pleiotropy in multiple disease resistance, based on the overlapping fine-mapping intervals, *pan1* mutants with phenotypic differences for two diseases, and UniformMu lines selected for resistance to NLB and potentially SLB. I initially selected loci for fine-mapping that

were known to provide protection against multiple pathogens. *pan1* emerged as a candidate gene for NLB and has been shown here to be a susceptibility gene for both NLB and Stewart's wilt. A complementation test is needed to determine whether *pan1* is underlying these two QTL. The fine-mapping interval for NLB and Stewart's wilt resistance at 1.06 is the most promising locus to be conditioned by a gene with pleiotropic effects. The fine-mapping confidence intervals for both diseases overlapped and the QTL peaks co-localized. The stronger Stewart's wilt effect may be reflected in the narrower confidence interval, while the weaker NLB effect may cause the fine-mapping interval to be larger. Re-sequencing of the Stewart's wilt interval is needed to determine the genic content of Tx303, the resistance donor, in this region. Community resources, including re-sequencing and annotation of diverse lines, that provide more insight into the genomic structure of other diverse lines would be helpful in dissecting complex traits in complex genomes.

Identifying genes associated with resistance

Based on my experiences fine-mapping, I would recommend an alternate approach for identifying genes associated with resistance. While fine-mapping provides strong evidence for candidate genes, it is ideal for QTL with high LOD scores and regions with high recombination rates. For many of these NLB resistance loci, these conditions are not met, requiring a different approach. Genome-wide association studies reveal many candidate genes and those can be followed up on with expression studies and mutant analysis.

A large number of candidate genes are being generated through association

mapping using different germplasm. For the most part, these candidates have not been compared across studies or independently validated. In order to take full advantage of these studies, findings must be curated. A meta-analysis of current studies would provide a mechanism by which to select the best candidates for further study. Those associations with the strongest statistical support from one panel could still be false positives or an aggregation of statistical support from linked genotypes. Selecting only a few candidates from association studies remains a challenge for this reason and alignment of different lines of evidence (i.e. other QTL studies, transcriptomics, metabolomics, etc.) is crucial to selecting candidates for validation.

Increasing the difficulty of validating candidate genes from association mapping is that association results vary from one analysis to another. Linkage and fine-mapping may also disagree with association mapping results. This was the case with *rik*. Screening of additional recombinants and an association re-analysis with updated kinship matrix refuted *rik* as a candidate gene. However, *rik* is up-regulated in response to *S. turcica* and there is strong literature support. It is possible that *rik* may be important in other populations, but not in this fine-mapping population.

Transcriptomics, even more powerful when combined with GWAS, can identify and validate genes involved in a trait. For example, exploring the differences in gene expression between the NILs would provide insight into what genes in the introgression may be modulating the resistance phenotype, as well as other genes and processes that are influenced by the QTL of interest. The data may be difficult to interpret however, as many genes may be differentially expressed. The availability of mutants for a significant number of genes in the maize genome makes this combined

approach of GWAS, transcriptomics, and mutant analysis attractive. The challenge in validating genes will be the increasingly small effects of each individual gene. Superior phenotyping methods may be needed to confirm the role of genes with small effects on disease phenotypes. More exact phenotyping may also improve the accuracy of association studies.

Breeding for disease resistance

Different breeding strategies are used at different stages of a breeding program. Genomic selection can be used early in a program to remove lines with low potential. Phenotypic selection can be used to develop improved lines. Meanwhile, marker-assisted selection can be used to incorporate a QTL for disease resistance at the finishing stage. Information about these loci could be applied during genomic selection to ensure that favorable alleles are in the population. The resolution of these fine-mapping studies narrows the loci sufficiently that marker-assisted selection could be applied for these loci.

Recently genomic selection has received a great deal of attention for its potential to accelerate breeding efforts and select for traits with low heritability (Heffner *et al*., 2009). There are limitations in the applicability of GS models across diverse environments and the technique is still under evaluation to determine its best use. In the case of breeding for drought tolerance, prediction was used to select for anthesis-silking interval to remove lines with poor performance (Cooper *et al*., 2014). A match must be struck between using genomic selection, phenotypic selection and integrating previous, including QTL, knowledge. This depends on matching

objectives, resources, and inheritance patterns.

Plant pathology

The field of plant immunity is advancing, but there has been limited integration between plant breeding and genetics and plant immunity research. Additionally, integration is needed between seed systems, agronomic management, and the use of crop diversity to advance crop protection strategies. Progress is being made in identifying effectors and using that information to identify corresponding R genes providing protection (Vleeshouwers 2011). Identifying suites of core effectors using next-generation sequencing and subsequent identification and stacking of R genes holds promise to incorporate durable resistance into crops (Dangl *et al*., 2013). Plant immunity and systems biology research are providing insight into multiple disease resistance and showing that highly connected proteins are targets effectors, as well as hormone-related genes (Mukhtar *et al*., 2011; Todesco *et* al. 2010).

Future directions

While I have made significant progress in identifying and validating candidate genes, further validation is required. The molecular mechanisms of resistance are not apparent from putative functions based on computational annotations; cell biology investigations are needed. Function of the genes must be confirmed. The histopathology and transcriptomics I have been involved in are important to understanding the molecular mechanisms. Integrating 'omics' data into a systems level understanding will also deepen the understanding of the disease resistance system.

Understanding the evolutionary dynamics surrounding these QTL would also be a significant contribution towards understanding the co-evolution of plant and pathogen and might provide insights into the durability of these loci.

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APPENDIX
FINE-MAPPING OF *HT2*

Introduction

Both quantitative and qualitative resistance has been mapped for northern leaf blight (NLB) caused by *Setosphaeria turcica.* A number of major genes have been described, including *Ht1*, *Ht2*, *Ht3*, and *HtN* (Hooker 1963, 1981; Hooker *et al.,* 1964; Hooker and Tsung 1980; Hooker 1977; Raymundo *et al.,* 1981). *Ht1* has been mapped to the smallest interval, having been narrowed to an 18 kb region containing two candidate genes (Wilson *et al.,* 2010). In another study, an NB-LRR gene cluster has been suggested to underlie the major gene *Ht1* (Martin *et al.,* 2011).

Multiple QTL studies have mapped NLB resistance to bin 6 of chromosome 8 (8.06) (Wisser *et al.,* 2006). The nested association mapping (NAM) populations identified two QTL at 8.06 and by genome-wide association mapping led to identification of hits in the 8.06 region (Poland *et al.,* 2011). One NAM GWAS hit was within the fine mapping interval (Poland *et al.,* 2011). It is located at 150,923,689 (RefGen_v1), which is between ctg358-37 and ctg358-44 in the *Ht2* fine-mapping interval.

The 8.06 region is known to harbor major genes, including *Ht2* and *HtN*. The original source of *Ht2* is NN14B. *Ht2* has been localized to 8.06 by previous fine-mapping studies (Chung *et al.,* 2010). Using a DK888 x S11 HIF population, Chung *et al.* (2010) were able to narrow the fine-mapping interval to 0.46 Mb, which contained 12 genes. A total of 47 recombinants in the 0.46 Mb region were identified, but a lack of marker density did not allow for further narrowing of the region. Since this

publication, genotyping-by-sequencing (GBS) has proven useful in identifying polymorhpisms in lines lacking extensive publical marker data (Elshire *et al.,* 2011). GBS was used in this study to identify SNP markers and further narrow the region using the recombinants across the 0.46 Mb region.

The main objectives of this study were to further narrow the physical region constituting *Ht2* using new markers developed from the GBS of the HIFs and to examine whether the DK888 genome is co-linear with the B73 genome in the fine-mapping region.

Materials and Methods

Genotyping

GBS was performed on samples derived from recombinants from the DK888 x S11 fine-mapping population. DNA was extracted using a Qiagen DNA extraction kit and GBS performed as previously described Elshire *et al.* (2011). Included in the samples for GBS were 33 DK888 x S11 recombinants, NN14B (a donor of *Ht2*), A619*Ht2*, A619, Oh43*Ht2*, Oh43, Pa91*Ht2* and Pa91.

Breakpoint analysis

SNPs derived from GBS of the recombinants were filtered so that only markers with no missing data were included in the analysis. IP scores collected in Aurora in 2009 (Chung *et al.,* 2010) were used in the breakpoint analysis. Single marker regression was performed in R using R/qtl as previously described (Jamann *et al.,* 2014)

Candidate gene identification

Candidate genes were considered based on position within the refined fine-mapping interval.

Genome structure

Non-B73 GBS tags that genetically mapped to 8.06 were obtained, as well as a count of the number of times a given tag was found in a given line (Fei Lu, personal communication). Tag counts were obtained for the recombinants and differential lines. Genotypes were transformed into 0 indicating no tag counts and 1 indicating tag counts, and phenotypes were transformed into 0 indicating resistant and 1 indicating susceptible. The correlations between genotype and phenotype were then examined.

Results

Using SNP markers with no missing data on the recombinants, the region was narrowed to a 143 kb interval spanning from 152,113,104 to 152,256,255 bp on chromosome 8, as shown in Fig. 1. Within this interval, there are five genes, including a putative reticulon (GRMZM2G091973), an uncharacterized gene (GRMZM2G393150), a cytidylyltransferase (GRMZM2G092018), a retrotransposon protein (GRMZM5G882216), and a putative serine/threonine protein phosphatase (GRMZM2G518717). It is important to note that two protein kinases immediately upstream of the narrowed fine-mapping interval, which were emphasized in the original fine-mapping study (Chung *et al.,* 2010), were not within this narrowed interval. The putative cytidylyltransferase contained a significant intronic NAM

association with a BPP value of 1.

A strong correlation between GBS tags and phenotypes was found between a tag at 152,103,243 bp, just upstream of the region, as shown in Figure 2. A BLAST search of the 64 bp tag did not identify any similar sequences. The strong correlation indicates that there is most likely structural variation that underlies the major gene *Ht2*. Sequencing of the fine-mapping region is needed.

Discussion

Using GBS to increase genotypic density on a population derived from parents not included in the HapMap dataset demonstrates the usefulness of GBS. Also, the high genotypic density was sufficient to narrow the region significantly. Interestingly, two putative protein kinases (GRMZM2G164612 and GRMZM2G451147) that are homologs of Arabidopsis *suppressor of npr1-1*, *SNC4*, are just outside of the fine-mapping interval. This, along with an immediate adjacent protein kinase, were emphasized by Chung *et al.* (2010) as very strong candidate genes.

The phenomenon of presence/absence variation in maize is well-described and there are high levels of genomic variation, even between two temperate inbreds (Fu and Dooner 2002; Springer *et al.,* 2009). A lack of genome integrity at 8.06 has been noted in other populations (Kolkman, personal communication). It is quite possible that the resistance gene is not present in B73, the sequenced line from which the candidate gene list was compiled. With any further narrowing of the region that reduces the number of candidate genes by position, it needs to be considered that the gene may not be present in the B73 genome, and thus may be absent from the

candidate gene list. Supporting this hypothesis is the strong correlation of non-B73 genome tags that map to the region with the disease phenotype.

The region encompassing *Ht2* has been narrowed and non-B73 genetic material identified that associated with the resistance phenotype. In order to identify the genic content of the fine-mapping region in DK888, complete sequencing across the region is needed to identify genes that may be present in DK888 but absent in B73. GBS tags might be used as an anchor point for PCR amplification.

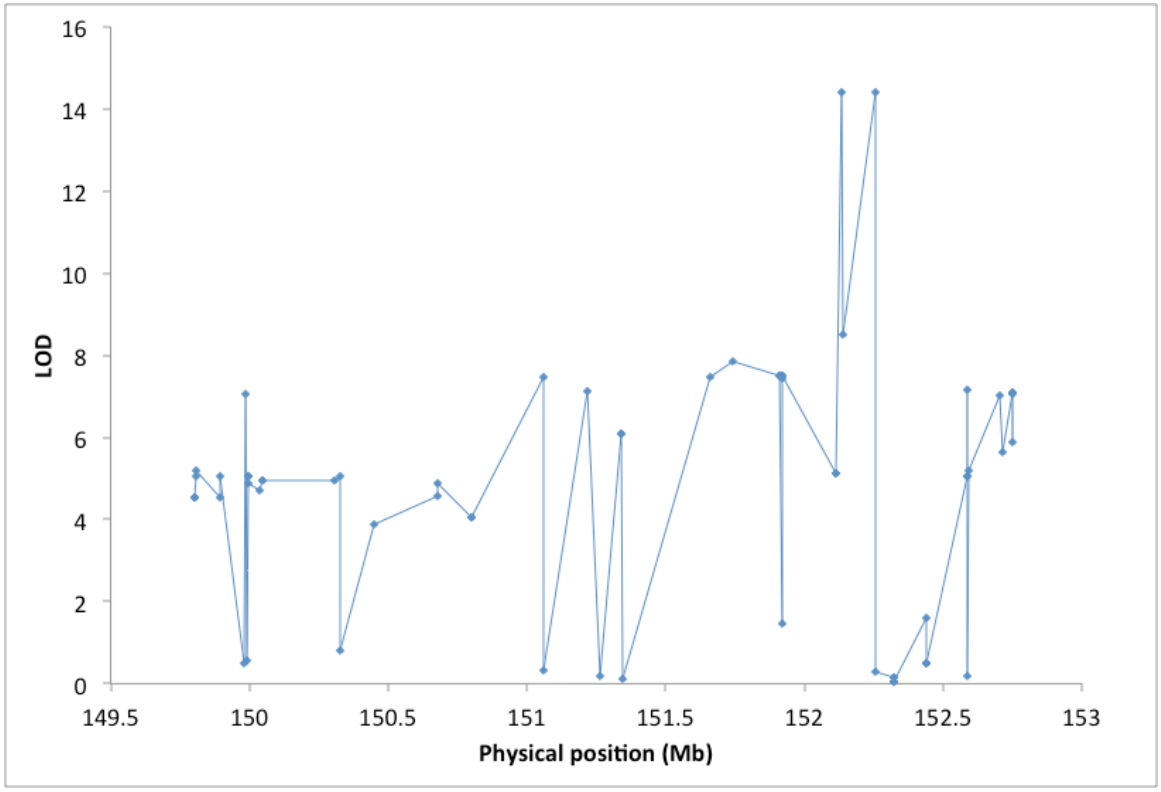

Figure 1. Fine-mapping. Fine-mapping results for *qNLB8.06*$_{DK888}$.

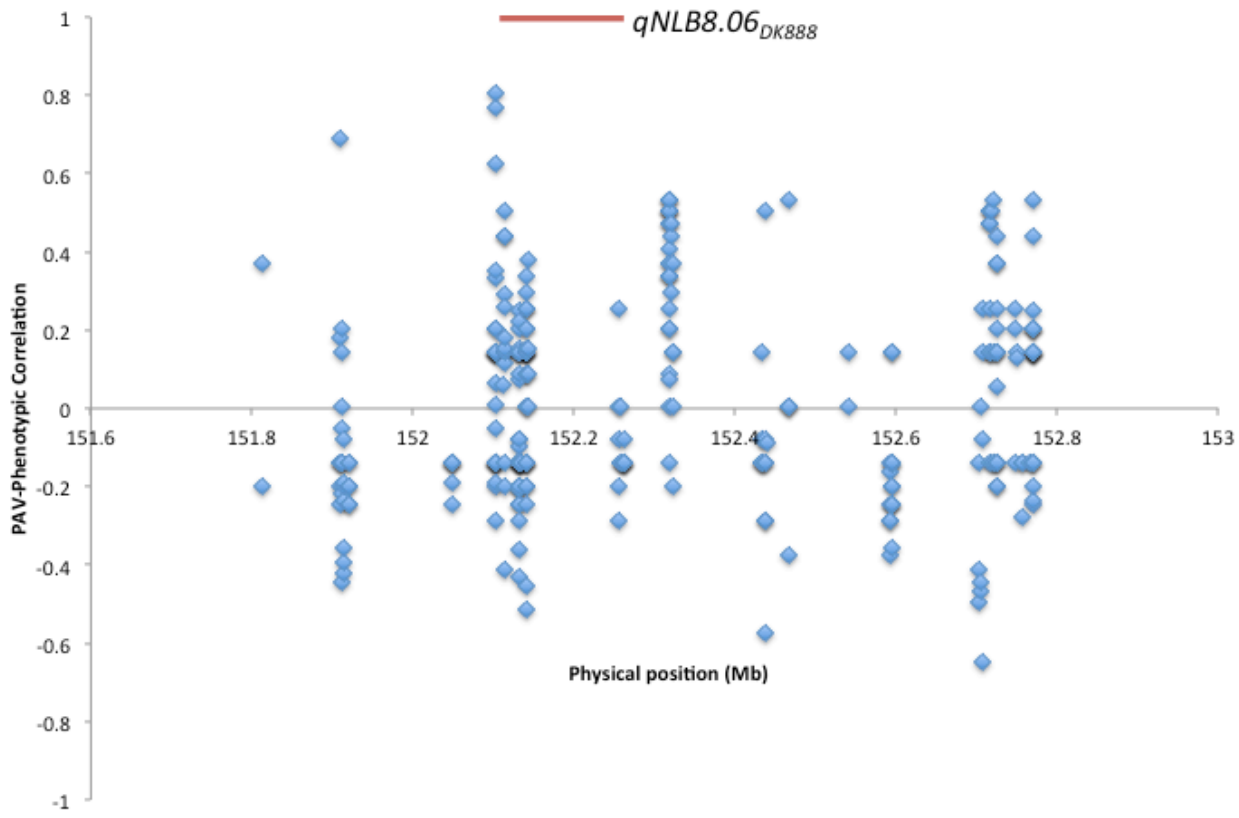

Figure 2. Presence/absence variation across the region of interest. A strong correlation was found between the presence or absence of non-B73 tags and disease phenotype in the DK888 fine-mapping population and the *Ht2* differential lines. The most highly correlated tag segregated nearly perfectly with resistance to NLB.

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